Systematics of subtribe Anthosperminae and the generic affinities of *Anthospermum* L. and *Nenax* Gaertn. (Rubiaceae: Anthospermeae)

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Signed this day ……**23**……of …**November**……..**2021**……..at ……**Bellville**……..

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ABSTRACT

The last taxonomic treatment of the subtribe Anthosperminae Benth. (Rubiaceae, Rubioideae, Anthospermeae) was in 1986 by Puff., nevertheless, few attempts have been made to resolve the phylogeny and the inter- and infrageneric relationships within the subtribe. The genera *Anthospermum* L. (39 species) and *Nenax* Gaertn. (11 species) are considered the most difficult groups to distinguish. *Anthospermum* species are widely distributed in Sub-Saharan Africa and Madagascar with the highest concentration of taxa in southern Africa, while *Nenax* species are restricted to southern Africa, in the south-western Cape Floristic Region. The two genera share common morphological and anatomical characters such as the growth form, presence of hairs on the stem, leaf arrangement, presence of petioles, flowers formation, dehiscence and presence of carpophore in fruits. currently combination of characters, woody shrub, needle-like leaves, few-flowered inflorescence and dioecy are considered unique in *Nenax*.

The most recent phylogenetic analysis based on molecular data indicated insights into generic relationships within the two genera and the subtribe Anthosperminae. The present study focussed on expanding the phylogenetic analysis of *Anthospermum*, *Nenax* and other genera within the subtribe, as well as assessing the value of selected morphological and anatomical characters for re-assesing generic circumscriptions. Phylogenetic relationships were analysed using Maximum Parsimony, Maximum Likelihood and Bayesian inference, and a Maximum Clade Credibility tree was

produced. These analyses were based on both nuclear (ITS, ETS) and plastid (*trnL-f*, *rps16*, *rpl32*) datasets.

Phylogenetic analyses of the combined nuclear and plastid (excluding *rpl32*) datasets, showed that the tribe Anthospermeae was strongly supported as monophyletic and sister to the tribe Putorieae, however, in the nuclear dataset the tribe Anthospermeae was sister to a paraphyletic clade comprised of members of the tribes Putorieae and Rubieae. Within the tribe Anthospermeae, Carpacocinae was resolved as sister to the remaining subtribes. A paraphyletic clade was recovered comprising of members of the subtribes Coprosminae and Opercullariinae. The subtribe Anthosperminae was strongly recovered as monophyletic, *Phyllis* L. and *Galopina* Thunb. formed sister groups to a strongly supported clade comprising of *Anthospermum* and *Nenax* species, and *Nenax* was embedded within *Anthospermum*.

Evolutionary patterns of 14 morphological characters within Anthosperminae were investigated by reconstructing these onto the Maximum Clade Credibility tree. This emphasized the extreme overlap of characters between *Anthospermum* and *Nenax*, and revealed no characters that could be used to define phylogenetic groups within the *Anthospermum* – *Nenax* clade.

This study based on morphological and molecular data showed the need to combine the genus *Nenax* and *Anthospermum* into one genus, *Anthospermum*, with 49 species recognized following an existing published revision. The combination of characters, such as small and narrow leaf blade, leaves without distinct petioles, fruits with small calyx lobes and mericarps separating into two with or without a carpophore or indehiscent mericarp without a carpophore, was revealed as important. A digital key

is also provided for southern Africa species, which includes images illustrating diagnostic characters for identifications and distribution maps.

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CHAPTER 1: GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

1.1 GENERAL INTRODUCTION

The Rubiaceae Juss. are comprised of approximately 13 000 species (Rova et al. 2002; Bremer and Eriksson 2009; Koekemoer et al. 2014) across 660 genera and is classified into 42 tribes (Yang et al. 2016). As such, the family is recognized as the fourth-largest family of flowering plants (Soza and Olmstead 2010a; Delprete and Jardim 2012; Rydin et al. 2017). Rubiaceae are cosmopolitan in their distribution, concentrated in the tropical or subtropical and temperate regions (Bremer and Eriksson 2009). The family displays an enormous variation in habit and growth form, including trees, shrubs and herbs, with the majority of the species being woody, and less than 20% of the genera herbaceous (Sonder 1865; Karou et al. 2011). Several well known pharmaceutical and agricultural crops are recorded from Rubiaceae, such as *Coffea canephora* Pierre ex A.Froehner. (Robusta Coffee), *Coffea arabica* L. (Arabica Coffee) and *Gardenia jasminoides* Retz. (Cape Jasmine) (Puff 1982; Karou et al. 2011; Zhao and Zhou 2020) to name a few.

Bremekamp (1954) recognized eight subfamilies based on testa structure, the occurrence of albumin in the seeds, presence or absent of raphides in the leaves, flowers, and fruit, and the ixoroid pollen mechanism (i.e., the pollen of protandrous flowers are discarded from the anthers onto the exterior of the swollen style before the flower is fully open and functional, and exposed for dispersal). Verdcourt (1958) reduced the number of subfamilies to only three (Rubioideae, Cinchonoideae and Guettardoideae) rejecting the pollen mechanism as a character to distinguish subfamilies, while noting the external hair type on the leaves and on the outer surface of

the flowers as important characters. Robbrecht (1988) recognized four subfamilies (Rubioideae, Cinchonoideae, Guettardoideae and Antirheoideae) based on the number of ovules, the presence of raphides in the floral parts, stem, leaves, or fruit, the absence of an endosperm and the presence of stylar pollen presentation. In addition, this classification system included more tribes than those in Verdcourt's (1958) classification. Phylogenetic analyses of Bremer et al. (1995), based on *rbc*L sequence data, corroborated three of the four subfamilies of the Rubiaceae (Robbrecht 1988), namely Cinchonoideae, Ixoroideae and Rubioideae, the fourth subfamily Antirheoideae received no support. Robbrecht and Manen (2006), based on *rbcL*, *rps16*, *trnL-trnF* and *atpB-rbcL*, recognized two subfamilies, Cinchonoideae and Rubioideae. However, Bremer and Eriksson (2009) produced a highly resolved tree, based on five plastid regions (*rbcL*, *trnT-F*, *rps16*, *atpB-rbcL*, *ndhF*), maintaining the division of the family into three subfamilies (Cinchonoideae, Ixoroideae and Rubioideae). This was also supported by Rydin et al. (2009a) when addressing deep divergences in the Rubiaceae family, utilising five plastid regions (*rbcL*, *trnT-F*, *rps16* intron, *atpB-rbcL* spacer, *ndhF*) and the internal transcribed spacer of the nuclear ribosomal DNA (nrITS1, 5.8S, nrITS2). Wikström et al. (2015) and Rydin et al. (2017), in their results based on five plastid regions (*rbcL*, *ndhF*, *matK*, *trnV*, *rps16*, *trnL-F*) also maintained the current three subfamilies Cinchonoideae, Ixoroideae and Rubioideae.

The tribe Anthospermeae Cham. and Schltdl. ex DC. forms part of the subfamily Rubioideae Verdc. and is largely distributed in South America, the South Atlantic islands, Africa and Australasia (Anderson et al. 2001). Members of the tribe are herbs, shrubs, dwarf shrubs or small trees distinguished by entire or divided stipules, unisexual flowers, stamens usually inserted at the base of the corolla, long and filiform

stigmas, as well as dry and dehiscent or fleshy fruits splitting into cocci or capsules (Chamisso and Schlechtendal 1828; Puff 1982; Bremer and Manen 2000). Puff (1982) divided the genera of the tribe into three subtribes: Anthosperminae Benth. (*Anthospermum* L., *Carpacoceae* Sond., *Galopina* Thunb., *Nenax* Gaertn. and *Phyllis* L.), Coprosminae Fosberg. (*Coprosma* J.R.Forst. & G.Forst., *Corynula* Hook.F., *Durringtonia* R.J.Hend. & Guymer., *Nertera* Gaertn., and *Normandia* Hook.f.) and Opercullariinae Benth. (*Eleuthranthes* F.Muell. ex Benth., *Opercularia* Gaertn. and *Pomax* Sol. ex DC.). In a study by Robbrecht (1982) pollen morphology (colporate or colpate grains), pollen shape and size within Anthospermeae and Paederieae supported the demarcation of genera by Puff (1982).

Members of the subtribe Anthosperminae are distributed in Africa, Madagascar, Macaronesia and Arabia, and characterized by their dry, dehiscent (rarely indehiscent) fruits, and unisexual or protandrous flowers (Puff 1982). The Coprosminae comprise of five genera with a trans-Pacific distribution, occurring in Australia, New Zealand, New Caledonia, Hawaii, Central America and South America (Thompson 2010). Coprosminae are characterized by drupaceous more or less fleshy fruits with a pair of planoconvex pyrenes and basally attached ovules (Thompson 2010). The Opercullariinae are restricted to Australia and Tasmania and are distinguished by umbellate inflorescences, protogynous (or unisexual) flowers and operculate fruits (Anderson et al. 2001).

In the first study to utilise molecular data by Bremer (1996), including only the *rbc*L region, the genera *Anthospermum, Coprosma, Nertera, Opercularia* and *Phyllis* were found embedded in a single clade, the Anthospermeae. Evidence from the *rps*16 intron in the analyses of Andersson and Rova (1999) revealed that the genera *Coprosma, Galopina, Leptostigma, Nenax, Nertera, Opercularia* and *Phyllis* formed a single clade.

Anderson et al. (2001), in their investigation of the relationships among Anthospermeae genera using ITS (ITS1 and ITS3) and the *rps*16 region, showed that the genera *Anthospermum*, *Coprosma*, *Nertera*, *Opercularia* and *Phyllis* were recovered in one clade sister to *Carpacoce*. However, Bremer and Eriksson (2009) in an attempt to delimit the tribe found *Carpacoce* strongly supported in a single clade within Anthospermeae similar to Bremer (1996). All the genera referred to as Anthospermeae by Puff (1982) were in a monophyletic group excluding all the genera referred to Paederieae in both studies by Bremer (1996) and Andersson and Rova (1999), which confirmed Puff's delimitation as well (Anderson et al. 2001). The most recent molecular study using five molecular markers from the plastid genome (*atpB-rbcL* intergenic spacer, *ndhF*, *rbcL*, *rps16* intron, and the *trnT-trnL-trnF* region) and two nuclear genome (nrETS and nrITS), by Thureborn et al. (2019) proposed a revised subtribal classification of the tribe Anthospermeae with a new monogeneric subtribe Carpacocinae Thureborn, Rydin & Razafim. Anthospermeae is therefore currently circumscribed with four subtribes (Anthosperminae, Carpacocinae, Coprosminae, and Opercullariinae) (Thureborn et al. 2019). In recent years, molecular data have shown some support for Puff's demarcation of tribes (Anderson et al. 2001). However, most studies used limited samples of taxa from each tribe and the phylogenetic relationships within Anthospermeae have also remained unclear (Thureborn et al. 2019).

Anthospermum L. (Anthosperminae) is a genus of 39 species of large shrubs, shrublets, short-lived subshrubs or perennial herbs largely concentrated in Africa south of the Sahara and in Madagascar, with the highest concentration of taxa in southern Africa (Puff 1982, 1986). The genus *Nenax* Gaerth. is a small genus of 11 species confined to the south-western and Western Cape, with one species extending into

Namibia (*Nenax coronata* Puff.) and another species widely distributed from the central-western Cape to the Free State and west of Lesotho (*Nenax microphylla* Sond.) (Puff 1986). The last comprehensive revision of these genera, which included nomenclature and revised taxonomic concepts, was conducted by Puff (1986). The two genera *Anthospermum* and *Nenax* are closely allied and the distinction between them is often unclear, with many of the characters overlapping and different species being confused with one another (Puff 1982, 1986; Thureborn et al. 2019). Currently the two genera are distinguished from each other through combination of characters, woody shrub, needle-like leaves, few-flowered inflorescence and dioecy are considered unique to *Nenax.* While *Anthospermum* have variable growth form, leaves large and broad to small and ericoid, inflorescence mostly congested and fruits always dehiscing into two merricarps (Puff 1986).

Thureborn et al.'s (2019) study included only half of the total number of the species within the two genera to determine their infrageneric relationships. In both the analyses of Bremer and Eriksson (2009) and Thureborn et al. (2019) there was poor taxon sampling and it was found that neither *Nenax* nor *Anthospermum* was monophyletic, and that *Nenax* accessions were recovered within *Anthospermum*.

The current study aimed to assess the phylogenetic relationships between *Anthospermum*, *Nenax* and related genera within the tribe Anthospermeae based on morphology and molecular data.

1.2 THE EXPLICIT OBJECTIVES OF THIS STUDY WERE TO:

1. Assess the phylogenetic relationships between *Anthospermum, Nenax* and other genera within the tribe Anthospermeae, through phylogenetic analysis of morphological and molecular sequence data.
2. Assess the generic circumscriptions of *Anthospermum* and *Nenax* using morphological and phylogenetic evidence.
3. Compile a fully illustrated and user-friendly electronic key to *Anthospermum* and *Nenax*.

CHAPTER 2: MATERIALS AND METHODS

Authorities for scientific plant names (according to Brummitt and Powell 1992) are given at first mention in each of the chapters and are also provided for each taxon in Appendix 1. All abbreviations utilized throughout this dissertation are given at first mention in each of the chapters.

2.1 MORPHOLOGICAL DATA

2.1.1 Taxon Sampling—Three field visits were conducted to collect material of species in the tribe Anthospermeae. A total of 49 samples (19 species) was collected. This material was processed to produce herbarium specimens housed at the Compton Herbarium (NBG). Species not collected from the field were sampled from herbarium specimens housed at Compton Herbarium (NBG). The voucher specimen information for these samples is listed in Appendix 1.

2.1.2 Examination of Herbarium Specimen—The complete collections of the genera of subtribe Anthosperminae (except *Phyllis* L.) housed at BOL and NBG (including SAM) were examined. In addition, 14 of 39 species of *Anthospermum* L. and 5 of 11 species of *Nenax* Gaertn. were studied *in situ* during field visits. Specimens from the herbaria, as well as those collected from the field, were used for examination of growth form, presence of hairs on the stem, leaf arrangement, presence of petioles, flowers (sexes, tubes, and stigma colour), and fruits (dehiscence, presence of carpophore).

2.1.3 Production of Electronic Delta Key—The DELTA Software package (Dallwitz et al. 1993) was used to produce a digital key to the species of *Anthospermum* and *Nenax*. Data from the literature Puff (1986) for consequently described species, as

well as from field observations were coded for each species in Delta Editor (Dallwitz et al. 1999). Using IntKey (Dallwitz et al. 1995) a digital key was produced from coded data and images from field collections of each species, INaturalist (https://www.inaturalist.org), JSTOR Global Plants (https://plants.jstor.org), as well as the diagnostic characters were added to the digital key. In addition, distribution maps of each species adapted from Puff (1986) were added to the key. The key is available at https://ranganin7.wixsite.com/anthospermum

This key provides a user-friendly tool for identification. The distinction between species may be troublesome, making the distinction of species difficult. The key contains images that aid in the identification of species, as well as images illustrating diagnostic characters. In addition, a digital key is advantageous, because the user can select the order in which to use characters for identification, rather than following a pre-established order, and the key provides substantial distribution maps of the species.

2.2 PHYLOGENETIC DATA

2.2.1 DNA Extraction, Amplification, and Sequencing—Total DNA was extracted from herbarium or fresh silica-dried leaf material (0.2 g) using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacture's protocol. Sources of plant material and voucher specimen information of the extracted material used during the study are listed in Appendix 1. GenBank accession numbers are also provided in Appendix 1 for those not extracted in this study but included in the analysis. Within the subtribe Anthosperminae a total of 29 (74%) species from *Anthospermum*, 4 (100%) species of *Galopina*, 9 (81%) species of *Nenax* and 2 (100%) species *Phyllis* were included in the phyloginatic analyses.

The primers used for polymerase chain reaction (PCR) amplification are provided in Table 1. PCRs were performed in 25 µl reactions containing: 12.5 µl EmeraldAmp GT PCR Master Mix [2X PCR Master Mix which composed of DNA polymerase, optimized reaction buffer, Deoxynucleoside triphosphate synthesis (dNTPs), density reagent and green dye] (Takara, Ohtsu, Japan); 1.0 µl Bovine Serum Albumin (BSA); 0.3 µl of both forward and reverse primers; 1.0 µl of dimethyl sulfoxide (DMSO) for nrDNA; 0.5 µl of DNA template and sterile distilled water to make up the final volume of 25 µl. Samples that showed difficulty in amplifying were diluted and the DNA template was also increased up to 4 µl, or these samples were cleaned using the OneStep™ PCR Inhibitor Removal Kit (Zymo Research) to remove contaminants from DNA preparations that were inhibiting PCR amplification. Zymo-Spin™ IV-HRC Columns were prepared by snapping off the base and removing the cap, inserted into a collection tube and centrifuged at 8,000 x g for 3 minutes. The amount of 50 µl DNA was transferred to a prepared Zymo-Spin™ IV-HRC placed in a 1.5 ml microcentrifuge tube and centrifuged again at 8,000 x g for 3 minutes. The purified DNA was used for PCR reactions.

The PCR reactions for nrDNA markers were carried out using the following times and temperature profile: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1 min, with final extension at 72°C for 8 min. Whereas the PCR reactions for cpDNA markers were carried out with the following times and temperature profile: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 8 min. For unsuccessful PCR samples, the procedure was adjusted to include a

temperature ramp following Shaw et al. (2005). Amplified PCR products were visualized using an ENDURO™ GDS Gel Documentation System. Successfully amplified PCR samples were cleaned using the ExoSAP PCR clean-up method of Werle et al. (1994) using 5 units of Exonuclease I (Exo) and 0.5 units of Shrimp Alkaline Phosphate (SAP). The PCR products were then sent to Macrogen (Seoul, Korea) for sequencing using the same primers used for PCR reactions.

Table 1. The primers used for Polymerase Chain Reaction (PCR) amplification and sequencing.

DNA region and primer name		Direction	Sequence (5'-3')	Reference
ITS:	AB101	Forward	ACG AAT TCA TGG TCC GGT GAA GTG TT	White et al. 1990
	AB102	Reverse	TAG AAT TCC CCG GTT CGC TCG CCG TT	White et al. 1990
	ITS2	Reverse	GCT GCG TTC TTC ATC GAT GC	White et al. 1990
	ITS3	Forward	GCA ECG ATG AAG AAC GCA GC	White et al. 1990
ETS:	ETS-ERIT	Forward	CTT GTA TGG GTT GGT TGG A	Baldwin and Markos 1998
	18S-ETS	Reverse	GCA GGA TCA ACC AGG TGA CA	Negrón-Ortiz and Watson 2002
rpl32:	*rpL32*-F	Forward	CAG TTC CAA AAA AAC GTA CTT C	Shaw et al. 2007
	trnL (*UAG*)	Reverse	CTG CTT CCT AAG AGC AGC GT	Shaw et al. 2007
rps16:	*rps16*F	Forward	AAA CGA TGT GGT ARA AAG CAA C	Oxelman et al. 1997
	*rps16*R	Reverse	AAC ATC WAT TGC AAS GAT TCG ATA	Oxelman et al. 1997
trnL-F:	*trnL*-c	Forward	CGA AAT CGG TAG ACG CTA CG-3	Taberlet et al. 1991
	*trn*L-f	Reverse	ATT TGA ACT GGT GAC ACG AG	Taberlet et al. 1991
	*trn*L-e	Forward	GGT TCA AGT CCC TCT ATC CC	Taberlet et al. 1991
	*trn*L-d	Reverse	GGG GAT AGA GGG ACT TGA AC	Taberlet et al. 1991

2.2.2 Phylogenetic Analyses—The sequences were aligned automatically using the Clustal W function in MEGA version 6.0. (Higgins et al. 1994; Tamura et al. 2013) and manual adjustment was done where necessary. Markers were analysed separately and in combination as well. The maximum parsimony (MP) algorithm was implemented in PAUP* version 4.0b4 (Swofford 2002). Character transformations were unordered and equally weighted (Fitch 1971). A heuristic search with 1000 random sequence additions, tree bisection reconnection (TBR) branch-swapping, and the MULPARS option selected, was performed for all analyses. All character transformations were treated with equal likelihood and a maximum of 10 trees were saved in each replicate to minimise time spent on swapping in each replicate. Trees of the shortest length were saved and used as starting trees for the second round of TBR swapping with no limit on the number of trees saved, to ensure the shortest trees were recovered in the analysis. Internal support was estimated with a search of 1000 bootstrap replicates (Felsenstein 1985) and a limit of 10 trees per replicate. The following scale was used to evaluate support percentages: 50%-74%, weak; 75%-84%, moderate; and 85%-100%, strong. Before combining the nuclear and plastid datasets, bootstrap consensus trees were compared by visual inspection to assess congruence of the separate datasets. These trees were considered incongruent only if they displayed 'hard' (i.e. with strong bootstrap support) rather than 'soft' (i.e. with weak bootstrap support) incongruence (Seelana et al. 1997; Wiens 1998).

Maximum likelihood (ML) analysis were executed using RAxML version 8.1.11 (Stamatakis 2006) on both combined plastid, nuclear and on combined plastid and nuclear datasets. The analysis was run on the CIPRES Portal, version 3.3 (Miller et al. 2010), using the default settings. The maximum likelihood trees with bootstrap node

support values are presented in the relevant chapter using the same scale used in MP analysis to evaluate support percentages.

Bayesian inference (BI) was implemented using MrBayes 3.2.3 (Ronquist and Huelsenbeck 2003). The analysis was run on the CIPRES Portal, version 3.3 (Miller et al. 2010). Data were partitioned accordingly in each dataset and all parameters were unlinked (statfreq, revmat, shape, pinvar) between partitions. The individual ITS, ETS, *rpl32*, *rps16* and *trnL-F* were analysed for 50 000 000 generations while combined datasets were analysed for 60 000 000 generations. The standard deviation of split frequencies stabilized below 0.01 for all analyses, providing evidence that a sufficient number of generations had been completed. Suboptimal trees were discarded as the "burn-in" phase (25%). only support values equal to and greater than 0.5 were retained, the following scale was used to evaluate support values: 0.50-0.94, weak; and 0.95-1.0, strong.

A Maximum Clade Credibility (MCC) tree for the selected species in the subtribe Anthosperminae, was performed using MCMC approach in MrBayes, PAUP was used to delete 25% "burn-in" of the number of generations, the results were summarized using TreeAnnotator which is part of the BEAST package on the CIPRES Portal, version 3.3 (Miller et al. 2010). MrEnt 2.5 (Zuccon and Zuccon 2014) was used to graphically display the MCC tree.

The best model and parameter estimates for Bayesian analysis were chosen using the jModeltest 2.1.10 (Darriba et al. 2012) on the CIPRES Portal, version 3.3 (Miller et al. 2010) (Table 2).

The outgroup species used to root the phylogeny were selected from Knoxieae, as they represent the closest related tribe to Anthospermeae, Spermacoceae and Rubieae (Bremekamp 1966; Andersson and Rova 1999; Anderson et al. 2001).

Table 2. The best model and parameter estimates for Bayesian analysis.

DNA region		AIC Model selected
Nuclear datasets:	ITS	GTR+I+G
	ETS	GTR+I+G
Chloroplast datasets:	*rpl32*	TPM1uf+I
	rps16	GTR+R
	trnL-F	TIM2+I+G

2.3 CHARACTER RECONSTRUCTION

Selected morphological characters that previously defined the genera were coded manually with a polarised outgroup (i.e. outgroup was coded as a "0" state) and reconstructed on Maximum Clade Credibility tree, using Mesquite version 3.04 (Maddison and Maddison 2015). 66 taxa were included. Character data were taken from field observations, herbarium specimens and literature (Puff 1982, 1986), selected characters are listed in Appendix 2.

CHAPTER 3: PHYLOGENETIC RELATIONSHIPS AND CHARACTER EVOLUTION IN *ANTHOSPERMUM* AND *NENAX* (ANTHOSPERMINAE, RUBIACEAE)

3.1 INTRODUCTION

Phylogenetic studies based on DNA sequence data have enhanced our understanding of evolutionary relationships of many plant groups, mainly because DNA yields more phylogenetic information than other sources of information (Brown 2002). Molecular techniques have been employed in many studies across plant groups and results found to be useful in taxonomic delimitation and determination of natural relationships of plant groups, especially those for which morphological data are ambiguous (Brown 2002; Susanna et al. 2006).

The utility of DNA sequences and morphological data in phylogenetic reconstructions have provided profound insights into the evolutionary relationships of the family Rubiaceae, and these have resulted in the identification of many lineages (Razafimandimbison and Bremer 2002; Robbrecht and Manen 2006; Bremer and Eriksson 2009; Delprete and Jardim 2012; Mouly et al. 2014). Many studies have applied molecular data to resolving systematic problems at all taxonomic levels (Manns and Bremer 2010).

While phylogenetic relationships in Rubiaceae were being studied (Bremer et al. 1995; Bremer 1996; Bremer and Manen 2000; Anderson et al. 2001; Andersson and Antonelli 2005; Robbrecht and Manen 2006; Bremer and Eriksson 2009; Rydin 2009a, 2009b; Manns and Bremer 2010; Krüger et al. 2012; Wikström et al. 2015; Rydin et al. 2017), attempts to resolve the phylogenetic relationships among and within the genera *Anthospermum* L. and *Nenax* Gaertn. are few (Anderson et al. 2001; Thureborn et al.

2019). As stated earlier (Chapter 1), of all available molecular reconstructions, none have included more than half of the total number of species in the genera *Anthospermum* and *Nenax* until recently (Thureborn et al. 2019).

Both *Anthospermum* and *Nenax* are currently placed within subtribe Anthosperminae, and the close relationship between the genera was indicated by previous studies (Anderson et al. 2001; Rydin et al. 2009b; Thureborn et al. 2019). However, the relationship of these genera to each other and other genera in the subtribe remains uncertain because neither *Anthospermum* nor *Nenax* were reported to be monophyletic (Thureborn et al. 2019). Thureborn et al. (2019) studied the phylogeny of the tribe Anthospermeae based on five plastid regions (*atpB-rbcL* intergenic spacer, *ndhF*, *rbcL*, *rps16* intron and the *trnT-trnF* region) and two nuclear regions (nrETS and nrITS), 25 species represented *Anthospermum* while *Nenax* was represented by five species.

Molecular markers, especially *rps16* intron, *trnT-trnF*, ITS and ETS, have often been used in the investigation of phylogenetic relationships in Rubiaceae (Anderson et al. 2001; Kårehed and Bremer 2007; Kårehed et al. 2008; Bremer and Eriksson 2009; Rydin et al. 2009b; Krüger et al. 2012). Sequence data from ITS and ETS are usually congruent with pseudogenes or recombinants not found, and as such combinable with plastid data to produce better phylogenetic resolution with higher support (Thureborn et al. 2019).

In this chapter, DNA sequence of two nuclear (internal and external transcribed spacers (ITS, ETS)) and three plastid genes (*trnL*-F, *rps16*, and *rpl32*) from *Anthospermum* and *Nenax* were added to Thureborn et al. (2019)'s phylogenetic data to address the infrageneric relationships between these two genera. Selected

morphological characters (such as habit, branching pattern, leaf arrangement, leaf size, presence of petioles, presence of pedicel, number of flowers per node, corolla merosity, dehiscent or indehiscent fruits, presence of carpophore and calyx lobes in fruits) were reconstructed onto the phylogeny to evaluate their pattern of character evolution in both genera, in order to assess their utility in generic circumscription.

The aims of this chapter are to:

1. Construct phylogenetic trees to study relationships between the genera *Anthospermum* and *Nenax*
2. Test the monophyly of these genera
3. Assess of the evolutionary history of relevant morphological characters used in generic circumscription in Anthosperminae

3.2 MATERIALS AND METHODS

All details of the materials and methods used are outlined in Chapter 2.

3.3 RESULTS

3.3.1. **rpl32** ***dataset—***The *rpl32* matrix consisted of a total of 829 unambiguously aligned positions resulting in 110 variable and 72 parsimony informative characters. The Maximum Parsimony (MP) analysis resulted in 2 197 trees, with a tree length of 209 steps, Consistency Index (CI) of 0.96 and Retention Index (RI) of 0.95 (Table 3). The topology of the MP strict consensus tree (Fig. 3.1) and Bayesian Inference (BI) majority rule (Fig. 3.2) were consistent with one another and with those presented by Thureborn et al. (2019).

The MP analysis was weakly resolved with several of the clades within the *Anthospermum-Nenax* clade moderately supported (Fig. 3.1), the resolution and support values are improved in the BI (Fig. 3.2) analysis.

In the BI (Fig. 3.2) analysis the tribe Anthospermeae was recovered as monophyletic with weak support (PP 0.67), while in the MP (Fig. 3.1) analysis the tribe Anthospermeae was recovered as paraphyletic, with *Carpococe* Sond. (BP 100) accessions sister to *Knoxia sumatrensis* (Retz.) DC. rather than grouping with the other members of the Anthospermeae.

Within Anthospermeae, the monogeneric subtribe Carpacocinae Thureborn, Rydin & Razafim. was weakly recovered as monophyletic in the BI analysis (PP 0.67) and is the most early diverging lineage within the tribe.

Subtribe Anthosperminae Benth. was strongly supported as monophyletic in both analyses (BP 100: Fig. 3.1; PP 1.00: Fig. 3.2). In all analyses, the *Anthospermum-*

Nenax clade was strongly supported (BP 100; PP 1.00). Two main clades (clade A and B) were recovered, although with moderate support in the MP (BP 71: clade A; BP 71: clade B), while clade A (PP 0.95) is strongly supported in the BI analysis and clade B (PP 0.63) weakly supported. In both analyses, *Anthospermum* and *Nenax* are recovered as polyphyletic with the species of *Anthospermum* and *Nenax* recovered in both main clades (clade A and B). The relationship of the two main clades remains the same in both analyses, with most of the *Nenax* species unresolved in clade B.

Nenax velutina J.C. Manning and Goldblatt and *Nenax coronata* Puff. were consistently recovered within clade A in both analyses. Clade A consists of most *Anthospermum* species including the generic type *Anthospermum aethiopicum* L.

Clade B comprised species from the genera *Anthospermum* and *Nenax* collected from the winter rainfall region (Western Cape) (BP 71: Fig. 3.1; PP 0.63: Fig. 3.2), while clade A comprised of species from the genera *Anthospermum* and *Nenax* collected from both winter and summer rainfall regions (Western Cape, Eastern Cape, Northern Cape KwaZulu-Natal, and Limpopo) (BP 71: Fig. 3.1 PP 0.95: Fig. 3.2).

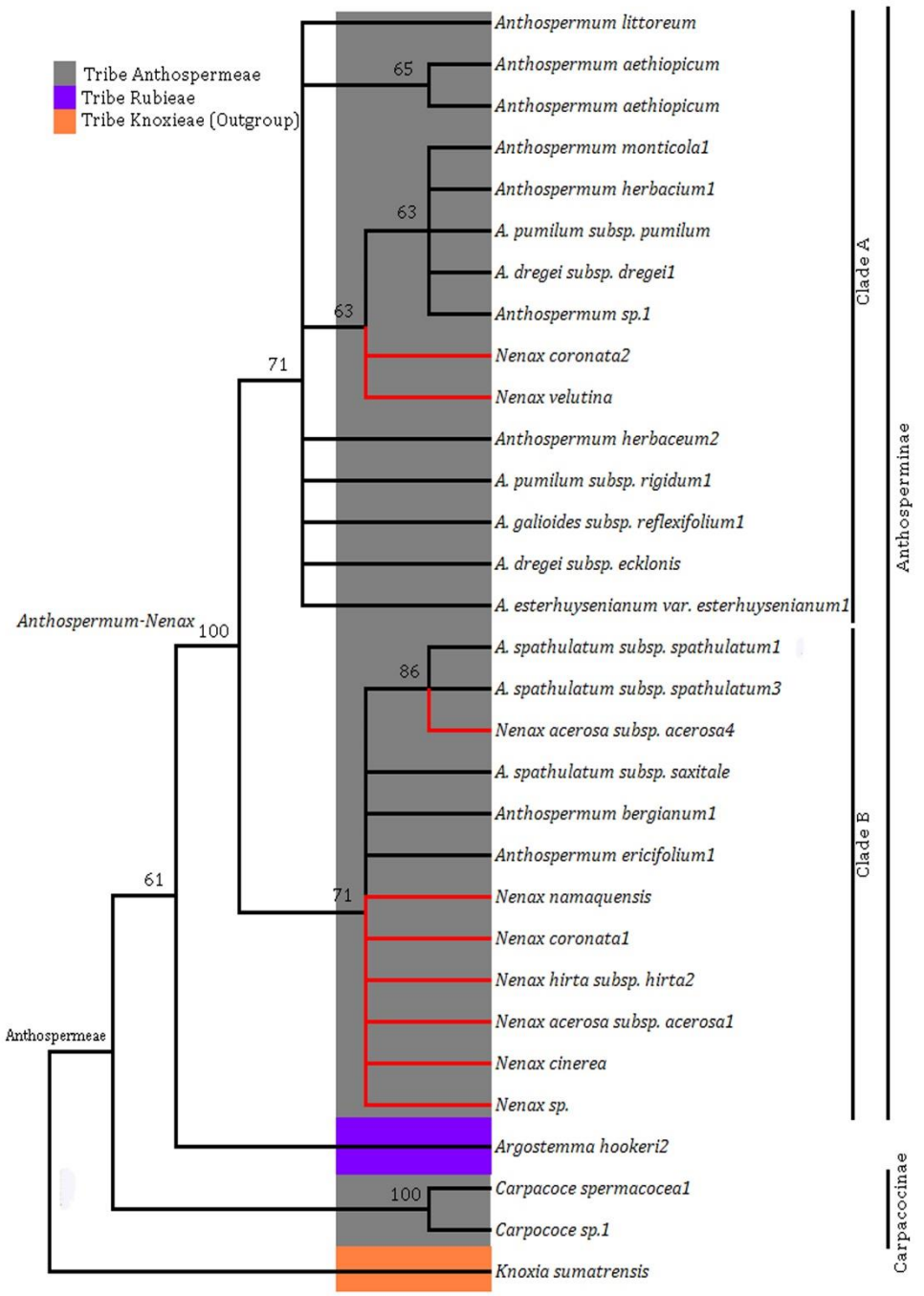

FIGURE 3.1. Bootstrap consensus tree from the Maximum Parsimony (MP) analysis based on the *rpl32* gene region, showing relationships within the tribe Anthospermeae. Bootstrap support (BP) values equal to and greater than 50% is indicated above the branches. Red branches indicate *Nenax* taxa.

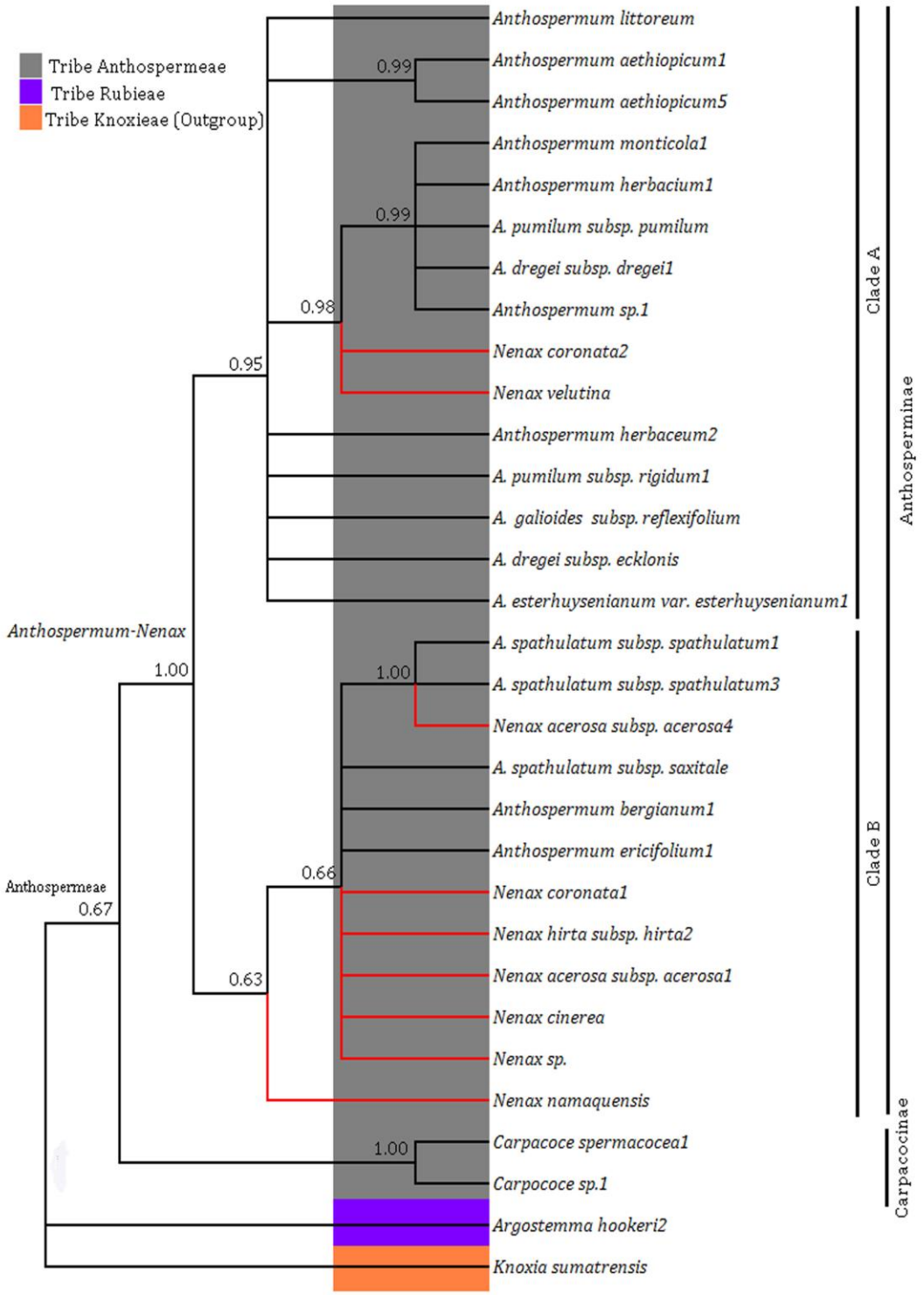

FIGURE 3.2. Majority rule consensus tree from the Bayesian Inference (BI) analysis based on the *rpl32* gene region, showing relationships within the tribe Anthospermeae. Posterior Probability (PP) values equal to and greater than 0.50 is indicated above the branches. Red branches indicate *Nenax* taxa.

The *rpl32* matrix was excluded from the combined datasets because Thureborn et al. (2019) did not utilize the *rpl32* gene region. This paper was published after commencement of the present study, and thus the gene regions do not overlap completely. Therefore, only 31 sequences representing 26 species for the genera *Anthospermum*, *Argostermma* Wall., *Carpococe*, *Nenax*, and *Knoxia* L. for *rpl32* were amplified for the present study.

***3.3.2 Combined plastid (*trnL-F *and* rpl16*) dataset*—** The plastid matrices comprised a total of 3 169 aligned positions with 481 variable and 791 parsimony informative characters. The parsimony analysis resulted in 660 582 trees with a tree length of 2 125 steps, CI of 0.78 and RI of 0.92 (Table 3). The topologies of the trees obtained from the MP strict consensus (Fig. 3.3) and the BI majority consensus tree (Fig. 3.4) were largely consistent with one another and with those presented by Thureborn et al. (2019).

The BI analysis (Fig. 3.4) was well resolved with moderately-/ strongly-supported clades and the resolution is better than in the MP analysis (Fig. 3.3).

The tribal topology with Knoxieae as outgroup in both BI and ML analyses were consistent, with the tribe Putorieae recovered as sister to the tribe Anthospermeae (BP 91: Fig. 3.3; PP 1.00: Fig. 3.4).

Anthospermeae was strongly recovered as monophyletic in both the MP and BI analyses (BP 91: Fig. 3.3; PP 1.00: Fig. 3.4). Within the Anthospermeae, the subtribe Carpacocinae was weakly recovered as monophyletic in both analyses (BP 62; PP 0.54) and is the earliest diverging lineage within the tribe. The Coprosminae Fosberg. was recovered as paraphyletic with *Normandia* Hook. F. sister to the remaining genera in the BI analysis (PP 1.00) and *Leptostigma* Arn. in the MP analysis (BP 78) rather than with

the other members of the Coprosminae (BP 58: Fig. 3.3; PP 1.00: Fig. 3.4). The Opercullariinae Benth. was strongly recovered as monophyletic in both the MP (BP 100) and BI (PP 1.00) analyses.

In both analyses, the subtribe Anthosperminae was recovered as monophyletic and sister to Coprosiminae with weak support in the MP analysis (BP 58) and strong support in the BI analysis (PP 1.00). Within Anthosperminae a clade comprising *Phyllis* L. (BP 99; PP 1.00) and *Galopina* Thunb. (BP 99; PP 1.00) was weakly recovered as the most early diverging clade in the BI (PP 0.88) and was strongly recovered in the MP (BP 98).

In both the MP and BI analyses the *Anthospermum-Nenax* clade was strongly supported (BP 99; PP 1.00).

Within the *Anthospermum-Nenax* clade, both *Nenax* and *Anthospermum* accessions were unresolved in one clade in the MP analysis (BP 99). However, in the BI analysis, three main clades (clade A, B and C) were recovered, although none received strong support and their relationships were unresolved. *Nenax* was recovered as polyphyletic with species recovered within all three of the main clades and embedded within *Anthospermum*. In the BI analysis *Nenax microphylla* (Sond) Salter. was recovered in its own clade, clade C (PP 1.00), and as the most early diverging clade.

The majority of the *Nenax* species, including the generic type (*N. acerosa* Gaertn.), were recovered within clade B together with some species of *Anthospermum* (*A. spathulatum* Spreng., *A. bergianum* Cruse and *A. ericifolium* (Licht. Ex Roem. & Schult.) Kuntze) in the BI analysis. The accessions of *Nenax* were largely recovered together in this clade although the position of *Nenax cinerea* (Thunb.) Puff and *Nenax divaricata* Salter was unresolved in the BI analysis.

Clade A in the BI analysis comprised most of the *Anthospermum* species, including the generic type (*A. aethiopicum*), although most species were unresolved together with the unresolved *Nenax velutina*, *Nenax coronata*, *Nenax namaquensis* Puff and *Nenax acerosa*. Clade C and B comprised of specimens collected in the winter rainfall region (Western Cape) and clade A comprises of specimens collected in both winter and summer rainfall regions (Western Cape, Eastern Cape, Northern Cape KwaZulu-Natal, and Limpopo) in the BI analysis.

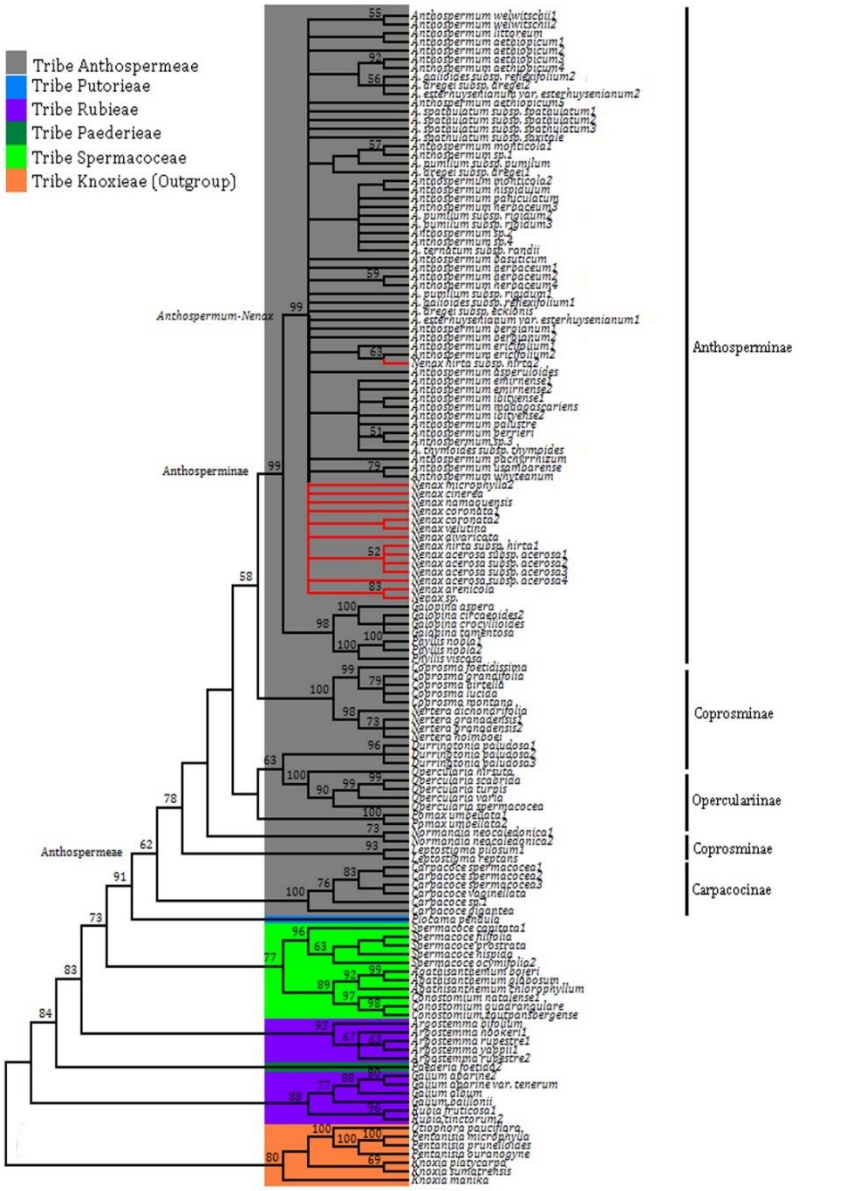

FIGURE 3.3. Bootstrap consensus tree from the Maximum Parsimony (MP) analysis based on the combined plastid (*trnL-F* and *rps16*) gene regions, showing relationships within the tribe Anthospermeae. Bootstrap support (BP) values equal to and greater than 50% are indicated above the branches. Red branches indicate *Nenax* taxa.

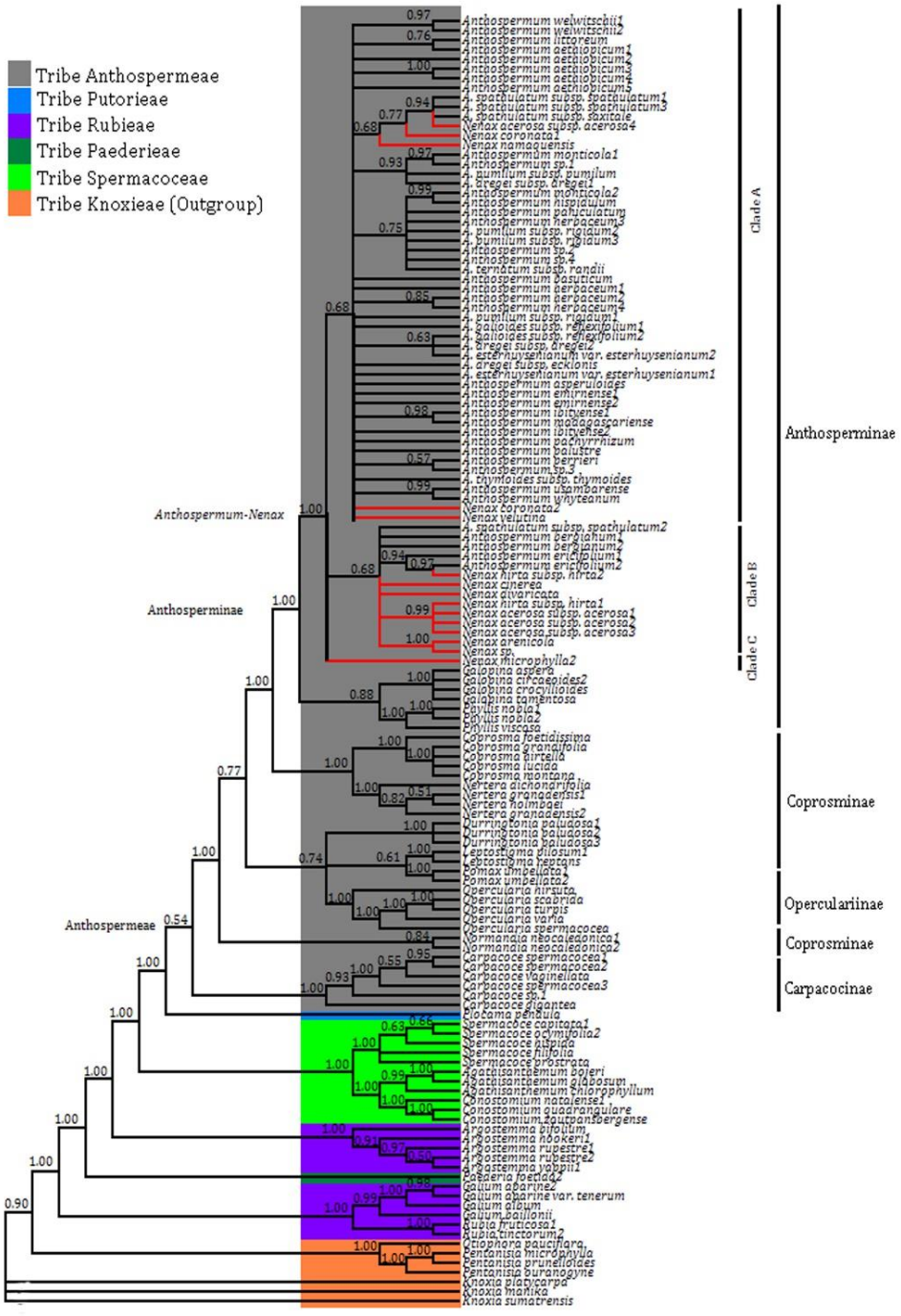

FIGURE 3.4. Majority rule consensus tree from the Bayesian Inference (BI) analysis based on the combined plastid (*trnL-F* and *rps16*) gene regions, showing relationships within the tribe Anthospermeae. Posterior Probability (PP) values equal to and greater than 0.50 are indicated above the branches. Red branches indicate *Nenax* taxa.

3.3.3 Combined nuclear (ITS and ETS) dataset— The combined nuclear matrix consisted of 1 331 aligned base pairs that included 160 variable and 631 parsimony informative characters. Maximum Parsimony (MP) analysis generated 174 157 trees with a tree length of 3 335 steps, CI of 0.44 and RI of 0.80 (Table 3). The phylogenetic trees produced from the combined nuclear datasets showed more resolution than the phylogenetic trees from the combined plastid dataset (Figs. 3.3-3.6). The topology for both the MP (Fig. 3.5) and BI (Fig. 3.6) analyses were consistent with one another and with those presented by Thureborn et al. (2019).

The MP analysis was only moderately resolved with several of the clades within Anthospermeae not supported (Fig. 3.5), the resolution and in some cases support values are improved in the BI (Fig. 3.6) analysis.

In the MP (Fig. 3.5) analysis, tribe Spermacoceae was recovered as sister to Rubieae, while in the BI (Fig. 3.6) analysis tribe Spermacoceae (PP 1.00) was recovered as sister to Paederieae (PP 0.99). In both the MP and BI analyses, Putorieae was embedded within Rubieae. The tribe Paederieae was recovered as sister to the tribe Anthospermeae in the BI analysis, and recovered as sister to Rubieae in the MP analysis, while Rubieae was also recovered sister to Anthospermeae in the MP analysis.

Anthospermeae was strongly recovered as monophyletic in the BI analysis (PP 1.00: Fig. 3.6) and recovered with no support in the MP (Fig. 3.5) analysis.

Within the Anthospermeae, the monogeneric subtribe Carpacocinae was strongly recovered as monophyletic in the BI analysis (PP 1.00: Fig. 3.6) and recovered with no support in the MP analysis (Fig. 3.5), Carpacocinae was the most early diverging lineage within the tribe. The Opercullariinae and Coprosminae were recovered together in a strongly supported clade in the BI analysis (PP 0.96: Fig. 3.6) and moderately supported

in the MP analysis (BP 75: Fig. 3.5). The Opercularinae and Coprosminae were both paraphyletic in the MP and BI analyses with *Opercularia* Gaertn. (BP 100: Fig. 3.5; PP 1.00: Fig. 3.6) sister to *Leptostigma* (BP 95: Fig. 3.5; PP 1.00: Fig. 3.6).

Subtribe Anthosperminae was strongly recovered as monophyletic (BP 99: Fig. 3.5; PP 1.00: Fig. 3.6) in all analyses and sister to the Opercullariinae-Coprosiminae clade (BP 75: Fig. 3.5; PP 0.96: Fig. 3.6). Within Anthosperminae a clade comprising *Phyllis* (BP 99) and *Galopina* (BP 100) was recovered as the earliest diverging clade in the MP analysis with no support, while strongly supported as sister to the *Anthospermum-Nenax* clade (BP 99). However, in the BI analysis, a clade comprising only *Phyllis* (PP 1.00) was recovered as the earliest diverging clade (PP 1.00), *Galopina* was weakly recovered as sister to the *Anthospermum-Nenax* clade (PP 0.66).

In all the analyses the *Anthospermum-Nenax* clade was strongly supported (BP 100: Fig. 3.5; PP 1.00: Fig. 3.6).

Within the *Anthospermum-Nenax* clade, *Nenax* was recovered as polyphyletic and embedded within *Anthospermum* in the BI analysis, while in the MP analysis *Nenax* was unresolved.

In the BI analysis *Anthospermum pumilum* subsp. *rigidum3* was recovered in its own clade (PP 1.00) and as the most early diverging clade. In the BI analysis *Nenax* species, including the generic type *N. acerosa* subsp. *acerosa,* were recovered within the *A. basuticum - A. littoreum* clade (PP 0.90). The accessions of *Nenax* were largely recovered together in the *N. namaquensis - A. spathulatum* subsp. *spathulatum* subclade (PP 0.64) within the *A. basuticum - A. littoreum* clade.

Within the BI analysis, the species of *Anthospermum* and *Nenax* recovered within the *A. basuticum* - *A. littoreum* clade were collected from both winter and summer rainfall regions. *Anthospermum* accessions such as *A. spathulatum* subsp. *spathulatum*, *A. spathulatum* subsp. *saxitale*, *A. aethiopicum* and *A. littoreum* were unresolved in both BI and MP analyses.

In both MP and BI analyses, the Madagascan and southern Tropical of Africa species of *Anthospermum* were recovered together within the *A. thymoides* – *A. welwetschii* clade (no support: Fig. 3.5; PP 0.87: Fig. 3.6), while clade *A. basuticum* - *A. littoreum* clade included both *Anthospermum* and *Nenax* species collected from southern Africa in the BI (PP 0.99) analysis.

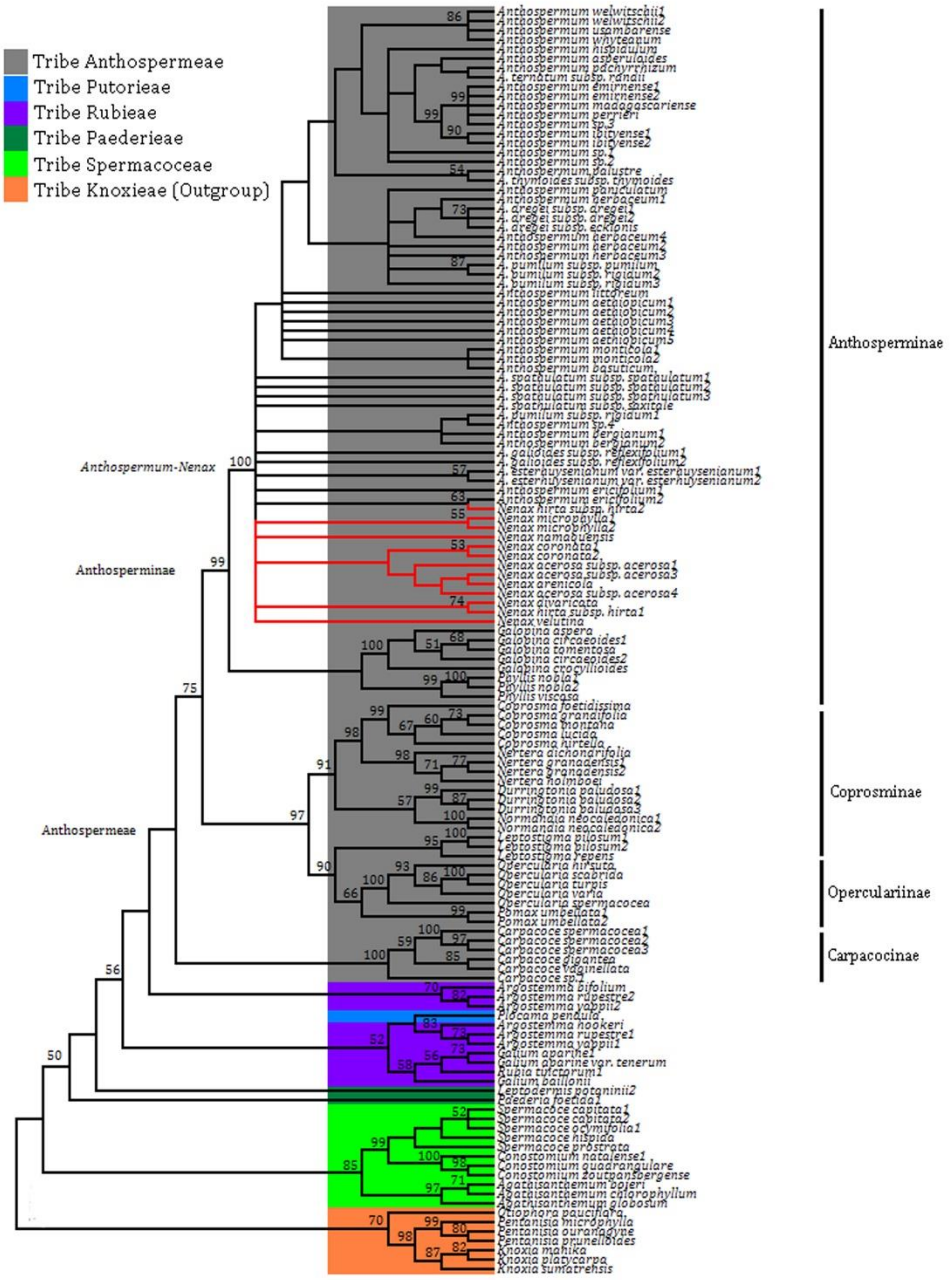

FIGURE 3.5. Bootstrap consensus tree from the Maximum Parsimony (MP) analysis based on the combined nuclear (ITS and ETS) gene regions, showing relationships within the tribe Anthospermeae. Bootstrap support (BP) equal to and greater than 50% are indicated above the branches. Red branches indicate *Nenax* taxa.

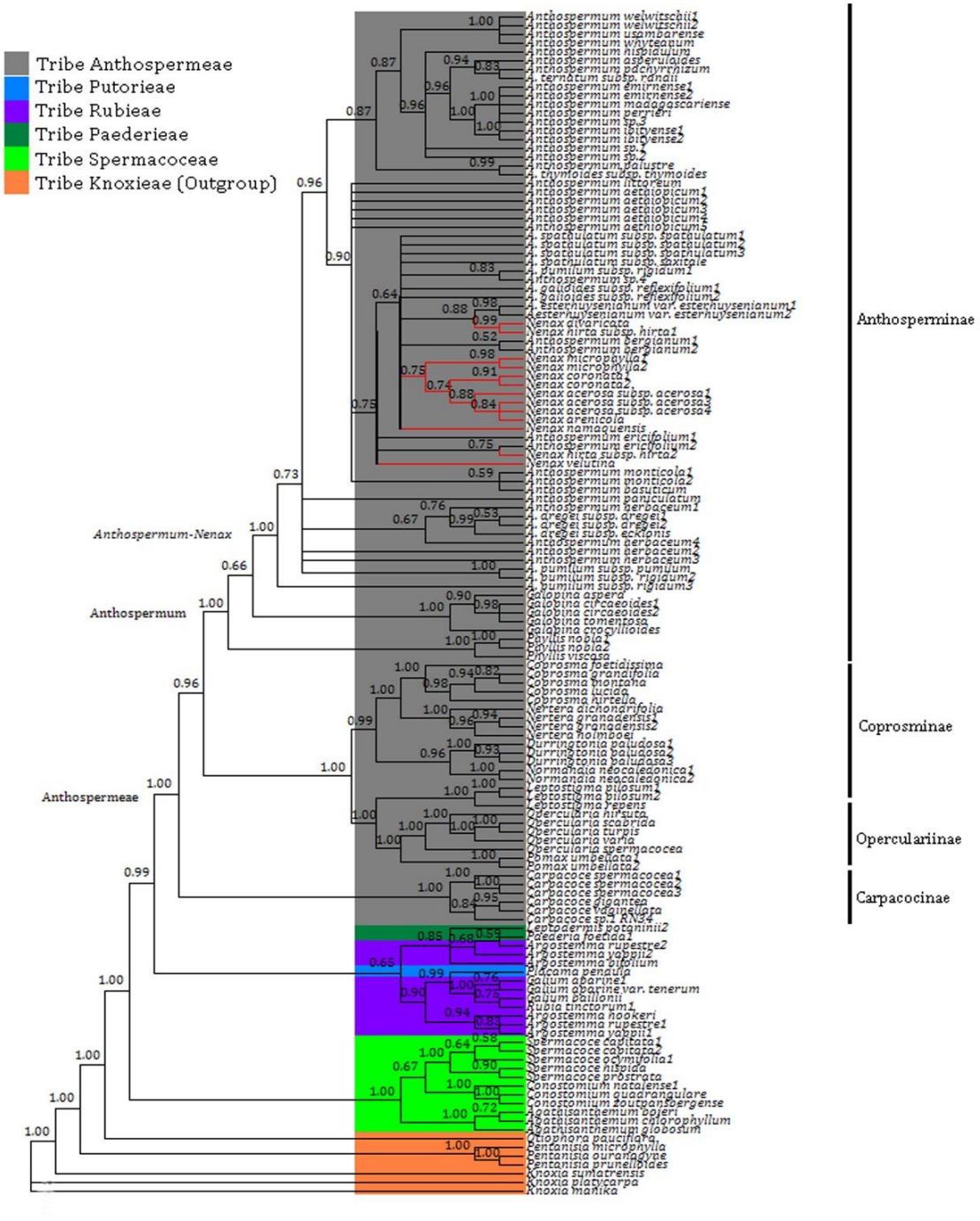

FIGURE 3.6. Majority rule consensus tree from the Bayesian Inference (BI) analysis based on the combined nuclear (ITS and ETS) gene regions, showing relationships within the tribe Anthospermeae. Posterior Probability (PP) values equal to and greater than 0.50 are indicated above the branches. Red branches indicate *Nenax* taxa.

3.3.4 Combined (*trnL-F/rps16*/ITS/ETS) dataset— Visual comparison of the separate nuclear and plastid analyses identified only weak incongruence between the two datasets and as a result, these datasets were combined directly. The combined matrix consisted of a total of 4 463 aligned positions resulting in 635 variables and 1 290 parsimony informative characters. The MP analysis recovered 466 857 trees, with tree length of 4 662 steps, CI of 0.61 and RI of 0.85 (Table 3). The topology of the MP strict consensus tree (Fig. 3.7), the BI majority consensus tree (Fig. 3.8) and Maximum-likelihood (ML) analyses (Fig. 3.9) were largely consistent with one another and with those presented by Thureborn et al. (2019).

While the MP analysis was only moderately resolved, with several of the clades within Anthospermeae not well supported (Fig. 3.7), the resolution and in some cases support values, were improved in the ML (Fig. 3.9) and BI (Fig. 3.8) analyses.

The topology of the outgroup tribes (Knoxieae, Spermacoceae, Rubieae, and Putorieae) in both BI and ML analyses were consistent while in the MP analysis the tribe Spermacoceae was embedded within tribe Knoxieae and Rubieae. In all the analyses tribe Putorieae was strongly recovered as sister to the tribe Anthospermeae (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8; BP 100: Fig. 3.9).

Anthospermeae was strongly recovered as monophyletic in both the MP and BI analyses (BP 100 PP 1.00) and moderately supported as monophyletic in the ML analysis (BP 84: Fig. 3.9).

Within the Anthospermeae, the subtribe Carpacocinae was strongly recovered as monophyletic in all three analyses (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8; BP 100: Fig. 3.9) and was the earliest diverging lineage within the tribe. The Opercullariinae and Coprosminae were recovered together in a strongly supported clade in all analyses (BP

100: Fig. 3.7; PP 1.00: Fig. 3.8; BP 98: Fig. 3.9). The Opercularinae and Coprosminae were both monophyletic in the MP and ML analyses (BP 99: Fig. 3.7; BP 98: Fig. 3.9 respectively) although in the BI analysis Coprosminae was recovered as paraphyletic with *Normandia* sister (BI 0.98) to a monophyletic Opercullariinae (BI 0.92) rather than with the other members of the Coprosminae (BI 1.00).

Subtribe Anthosperminae was strongly recovered as monophyletic (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8; BP 100: Fig. 3.9) in all analyses and sister to the Opercularrinae - Coprosminae clade (BP 99: Fig. 3.7; PP 1.00: Fig. 3.8; BP 98: Fig. 3.9). Within Anthosperminae a clade comprising *Phyllis* (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8) and *Galopina* (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8) was weakly recovered as the most early diverging clade in the MP and BI analyses (BP 63: Fig. 3.7; PP 0.56: Fig. 3.8). However, in the ML analysis, *Phyllis* was recovered as sister to the *Anthospermum* - *Nenax* clade, albeit with no support.

In all analyses the *Anthospermum* - *Nenax* clade was strongly supported (BP 100: Fig. 3.7; PP 1.00: Fig. 3.8; BP 100: Fig. 3.9).

Three main clades (clade A, B and C) were recovered, although without support in all analyses. *Nenax* was recovered as polyphyletic in all analyses with the species recovered within all three of the main clades and embedded within *Anthospermum*.

Nenax microphylla was consistently recovered in its own clade, clade C (BP 100, BP 100, PP 1.00), and was the most early diverging clade in the MP and ML analyses. The relationship of the three main clades was unresolved in the BI analysis.

The majority of the *Nenax* species, including the generic type (*N. acerosa*) were recovered within clade B together with several species of *Anthospermum* (*A*.

spathulatum subsp. *spathulatum*, *A. spathulatum* subsp. *saxatile*, *A. bergianum* and *A. ericifolium*). The accessions of *Nenax* were largely recovered together in this clade although the position of *N. namaquensis* was unresolved in the MP and BI analyses. The species of *Anthospermum* and *Nenax* recovered in clade B were from the winter rainfall region (BP 76: Fig. 3.7; PP 1.00: Fig. 3.8; BP 85: Fig. 3.9).

Most of the species of *Anthospermum*, including the generic type (*A. aethiopicum*), were recovered within clade A, together with *Nenax velutina*. Additionally, in the BI analyses one of the *Nenax coronata* accession was also placed in this clade, while in the MP and ML analyses both *Nenax coronata* accessions were recovered in clade B. Clade A was largely unsupported, receiving weak support only in the BI analysis (PP 0.50), and the relationships within this clade were largely unresolved with differing topologies between the different analyses.

The Madagascan species (*A. ibityense* – *A. emirnense* group, BP 100 PP 1.00 BP 99) were recovered together within clade A and placed within a subclade that includes species from southern Tropical Africa. *Anthospermum* species from both the summer and winter rainfall regions of southern Africa were included in this clade.

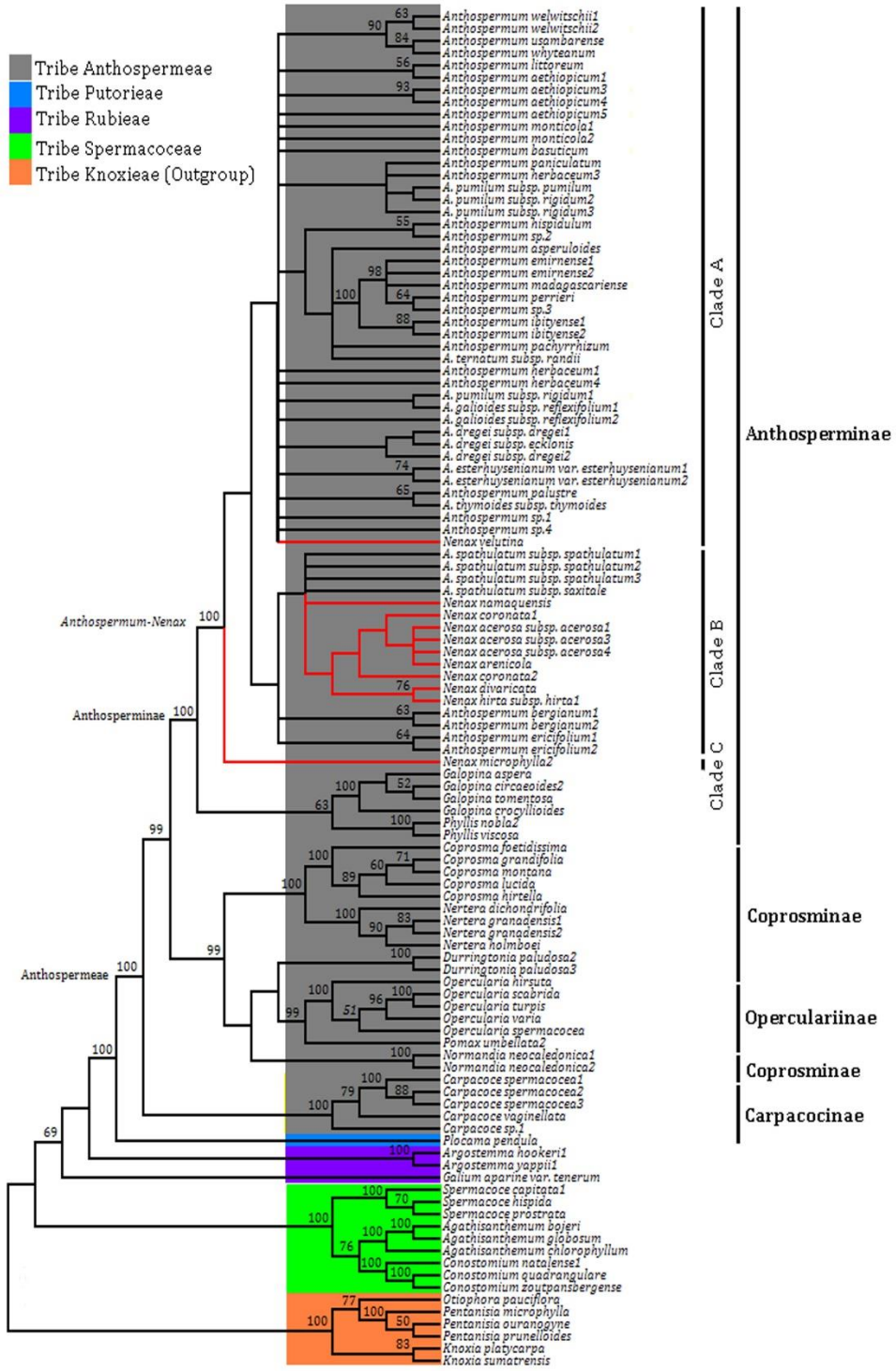

FIGURE 3.7. Bootstrap consensus tree from the Maximum Parsimony (MP) analysis based on the combined (plastid and nuclear) gene regions, showing relationships within the tribe Anthospermeae. Bootstrap support (BP) equal to and greater than 50% are indicated above the branches. Red branches indicate *Nenax* taxa.

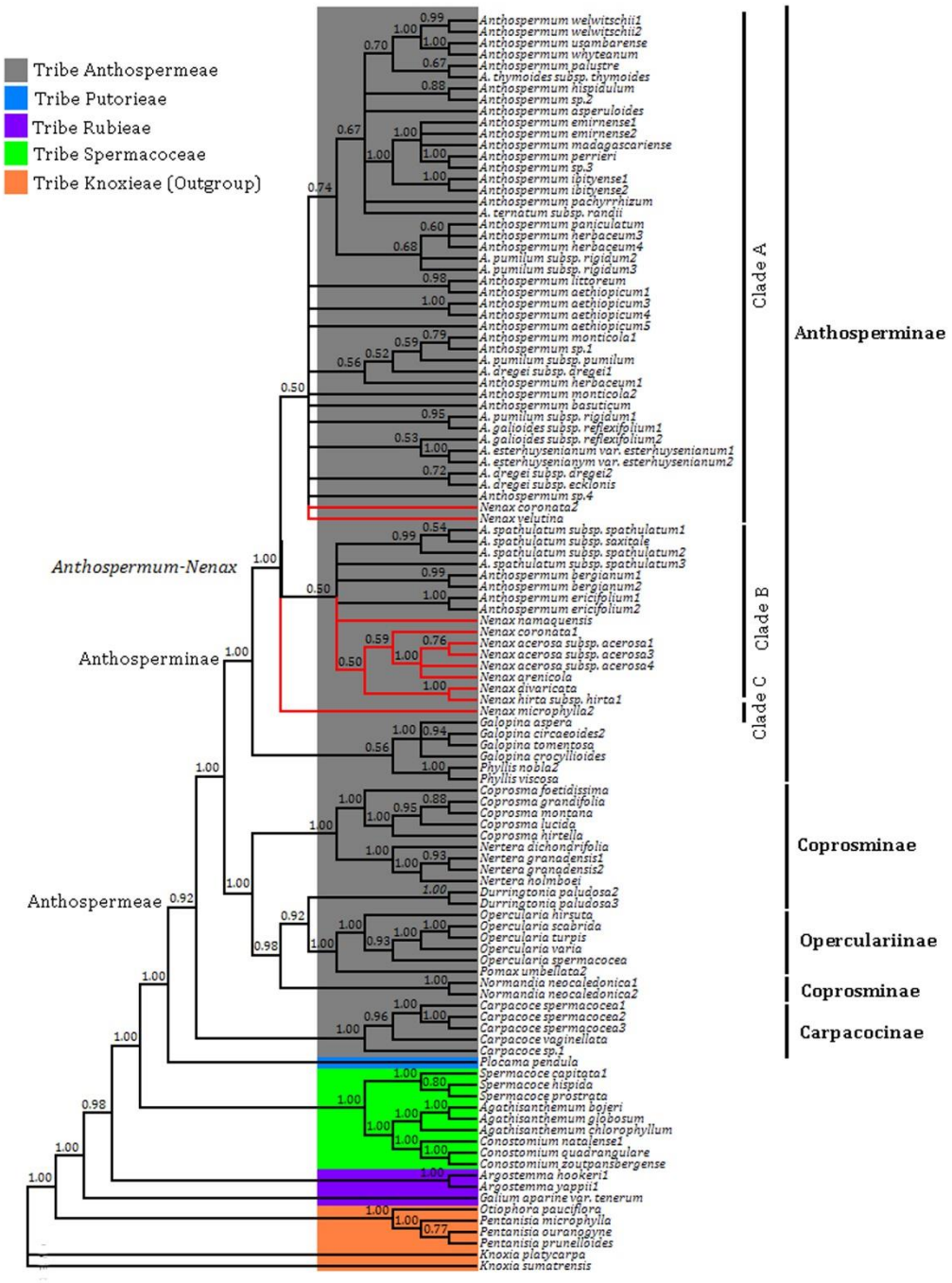

FIGURE 3.8. Majority rule consensus tree from the Bayesian Inference (BI) analysis based on the combined (plastid and nuclear) gene regions, showing relationships within the tribe Anthospermeae. Posterior Probability (PP) values equal to and greater than 0.50 is indicated above the branches. Red branches indicate *Nenax* taxa.

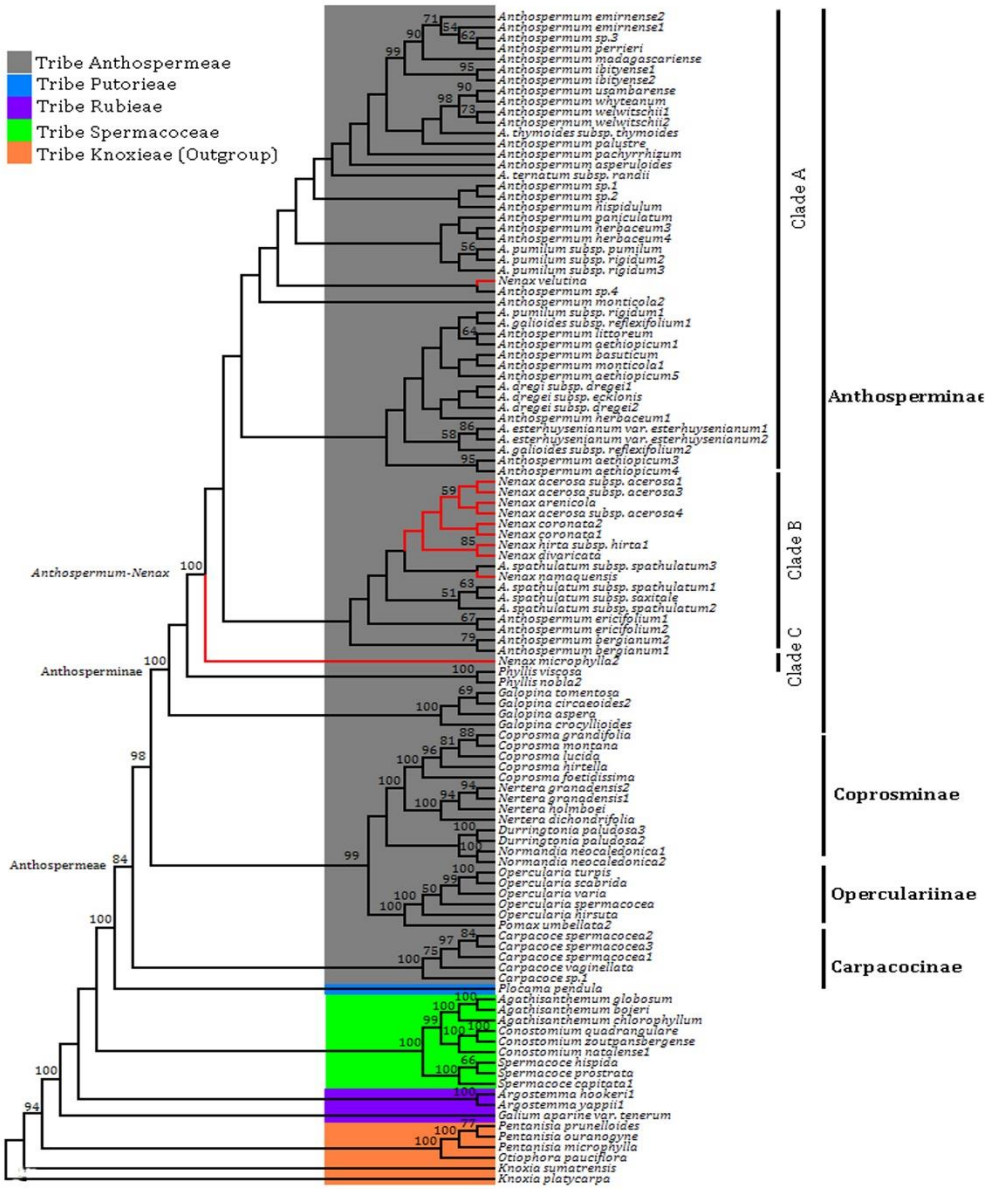

FIGURE 3.9. Maximum-likelihood tree based on the combined (plastid and nuclear) gene regions, showing relationships within the tribe Anthospermeae. Bootstrap support (BP) values equal to and greater than 50% are indicated above the branches. Red branches indicate *Nenax* taxa.

Table 3. Statistics and sequence characteristics of the plastid, nuclear and combined analyses of the Anthosperminae.

DNA region	***rpl32***	**Plastid** ***(trnl-F & rps16)***	**Nuclear** **(ITS & ETS)**	**Combined**
No. of taxa	31	135	135	111
No. of included positions in a matrix	829	3169	1331	4463
No. of constant characters	647	1897	540	2538
No. of parsimony informative characters	72	791	631	1290
No. of variables sites	110	481	160	635
No. of trees (Fitch)	2197	660582	174157	466857
Tree length	209	2125	3335	4662
Consistency Index (CI)	0.96	0.78	0.44	0.61
Retention Index (RI)	0.95	0.92	0.80	0.85
The average number of changes per variable site (number of steps/number of variable sites)	1.9	4.4	20.8	7.3

3.3.5 Evolution of morphological characters— The reconstructions of 14 morphological characters onto the Maximum Clade Credibility (MCC) tree (Fig. 3.10) followed the methods outlined in Chapter 2 Section 2.3 and are presented in Figs. 3.11 – 3.15. These trees only show subtribe Anthosperminae, focusing on *Anthospermum* and *Nenax*. The matrix of character states of selected species of the subtribe Anthosperminae is provided in Appendix 2.

Species of the genus *Nenax* have a woody subshrubby habit, but variation in habit is quite pronounced within *Anthospermum,* which is comprised of woody shrubs, subshrubs and perennial herbs while *Galopina* comprises of perennial herbs (Puff 1986; Fig. 3.16). The perennial herbs is regarded as a synapomorphy for *Galopina*.

In the present reconstruction, the erect, single stemmed habit was recovered as the plesiomorphic state within the subtribe Anthosperminae. The erect stems, trailing/prostate and mat-forming stems were found within *Anthospermum* species, and erect stems were also found in all *Nenax* species, except for *N. hirta* (Cruse) Salter with trailing stems (Fig. 3.11B). In all genera of Anthosperminae, the young stems are usually covered with hairs, however, in older woody stems of some *Anthospermum* and *Nenax* species, a thin layer of cork cells is formed due to phellogen originating from the subepidermal layer, and therefore hairs are no longer found in older stems (Puff 1986). The reconstruction of stems with hairs either short or long in this study was based on older stems. Stems with hairs were recovered in *Galopina* while *Anthospermum* and *Nenax* species comprised of both glabrous and hairy stems (Fig. 3.11C).

Within *Galopina* and the majority of *Anthospermum* and *Nenax* species, the leaves are arranged decussately (Puff 1986). Similarly, in the present reconstruction, decussate leaves were recovered as the plesiomorphic character state with leaves

arranged in whorls of three only recovered in *Anthospermum* and *Nenax* species (Fig. 3.12A).

The presence of hairs on both surfaces of the leaves was recovered as the plesiomorphic character state, with leaves covered only on the upper surface a derived character in *Anthospermum* species. The majority of *Anthospermum* and *Nenax* species were recovered with glabrous leaves. The presence of hairs on the leaves was recovered as a variable character in *Anthospermum* as it included both leaves with hairs on both surfaces, only the upper surface, and entirely glabrous (Fig. 3.12B).

Within Anthosperminae, *Galopina* has the largest and broadest leaves, however, *Anthospermum* species show great variation with large and small leaves (Puff 1986). Similarly, in the present reconstruction, *Galopina* was recovered with large and thin leaves, while *Nenax* and *Anthospermum* were recovered with small and narrow leaves, except for *A. welwitschii* Hiern. (Fig. 3.12C) that has large and thin leaves. *Galopina* has been known to have leaves with long petioles (Puff 1986). The presence of petioles was recovered as plesiomorphic, with the absence of petioles a derived character for some *Anthospermum* and *Nenax* species (Fig. 3.13A).

Flowers in paniculate to thysic, many-flowered inflorescences is considered the most common inflorescence type within the subtribe Anthosperminae (Puff 1986). In *Anthospermum* and *Nenax*, the majority of species commonly have subsessile or very shortly pedicellate flowers (Puff 1986), as seen in the reconstruction where sessile/subsessile flowers were recovered in *Anthospermum* and *Nenax* species except for *A. paniculatum*, *N. namaquensis*, *N. coronata* and *N. divaricate* (Fig. 3.13B). Both *Anthospermum* and *Nenax* comprised of species with a single flower or paired flowers or

in a cluster of many (more than two) flowers at the node, while single-flowered species were recovered as plesiomorphic for *Galopina* (Fig. 3.13C).

Within the subtribe Anthosperminae, the most common corolla merosity is 4-merous, but there are odd occurrences of 5-merous flowers (Puff 1986). In this study the 5-merous derived character was recovered within the majority of *Nenax* species and a few species of *Anthospermum* (Fig. 3.14A). The 4-merous flowers were recovered as the ancestral state for both *Galopina* and the *Anthospermum*-*Nenax* clade (Fig. 3.14A).

The fruits of all *Anthospermum* and some of *Nenax* species were recovered with the presence of a carpophore, however, the majority of *Nenax* species and all *Galopina* species were recovered with the carpophore absent (Fig. 3.14B). Dehiscent fruits were recovered as the plesiomorphic character state, with all taxa of *Anthospermum* and *Galopina* having dehiscent fruits, while *Nenax* has species with dehiscent and indehiscent fruits (Fig. 3.14C). Fruits without calyx lobes were recovered as plesiomorphic for the *Galopina* species and as a derived character for *Anthospermum* species. All *Nenax* species and the majority of *Anthospermum* species were recovered with the presence of calyx lobes on the fruits (Fig. 3.15A).

The *Anthospermum*-*Nenax* clade showed variation of the flowering season such as winter, autumn, summer and, the most dominant, spring among *Anthospermum* species, while summer is the flowering season for all *Galopina* species (Fig. 3.15B).

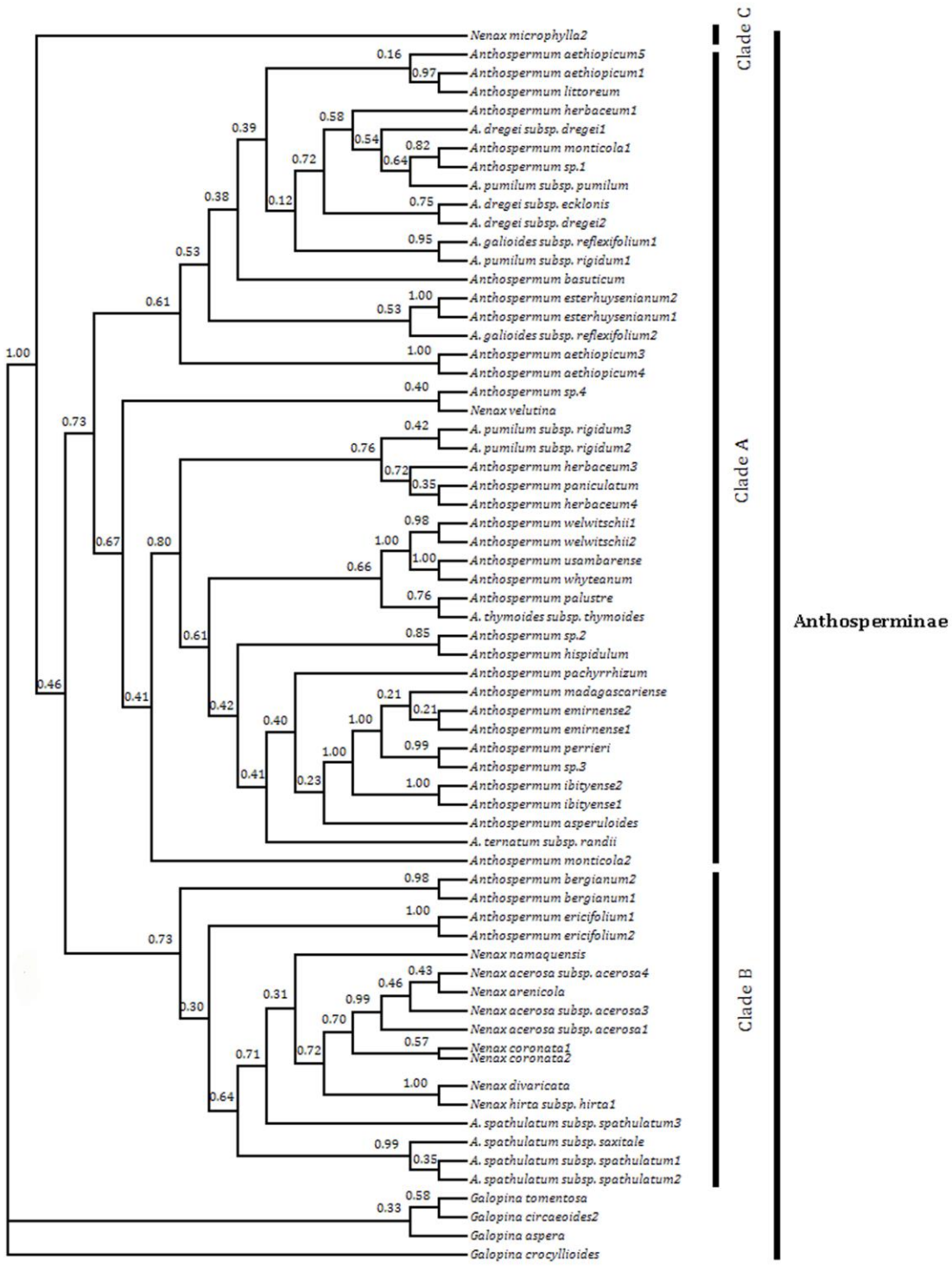

FIGURE 3.10. Shortened version of the Maximum Clade Credibility (MCC) tree based on the combined (plastid and nuclear) gene regions, showing relationships within the subtribe Anthosperminae. Bootstrap support (BP) values equal to and greater than 50% indicated above the branches.

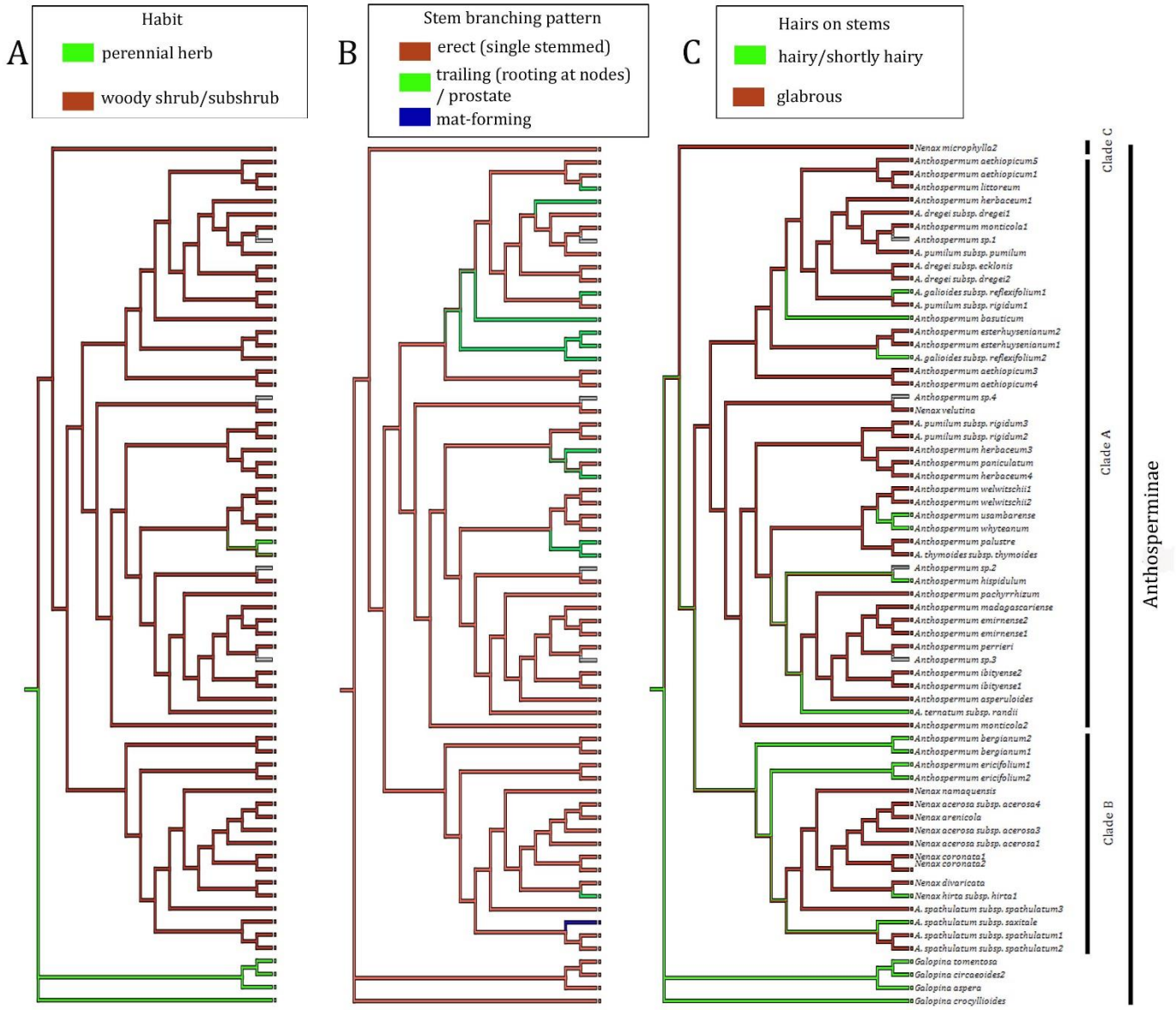

FIGURE 3.11. Character reconstruction of habit (A), stem branching pattern (B) and hairs on the stem (C) in the subtribe Anthosperminae, mapped onto the Maximum Clade Credibility tree, produced from the combined dataset.

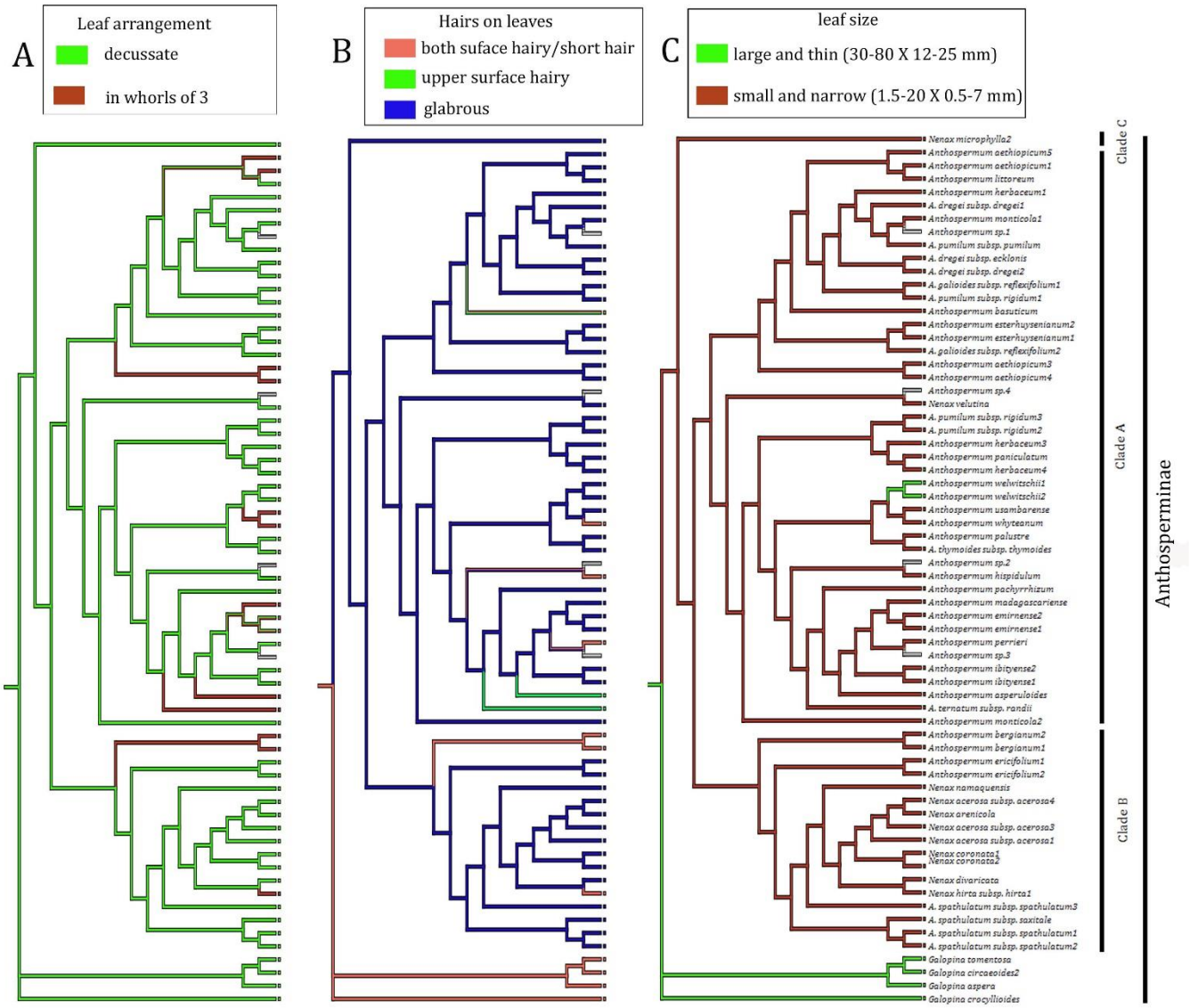

FIGURE 3.12. Character reconstruction of leaf arrangement (A), hairs on the leaves (B) and leaf size (C) in the subtribe Anthosperminae, mapped on the Maximum Clade Credibility tree, produced from the combined dataset.

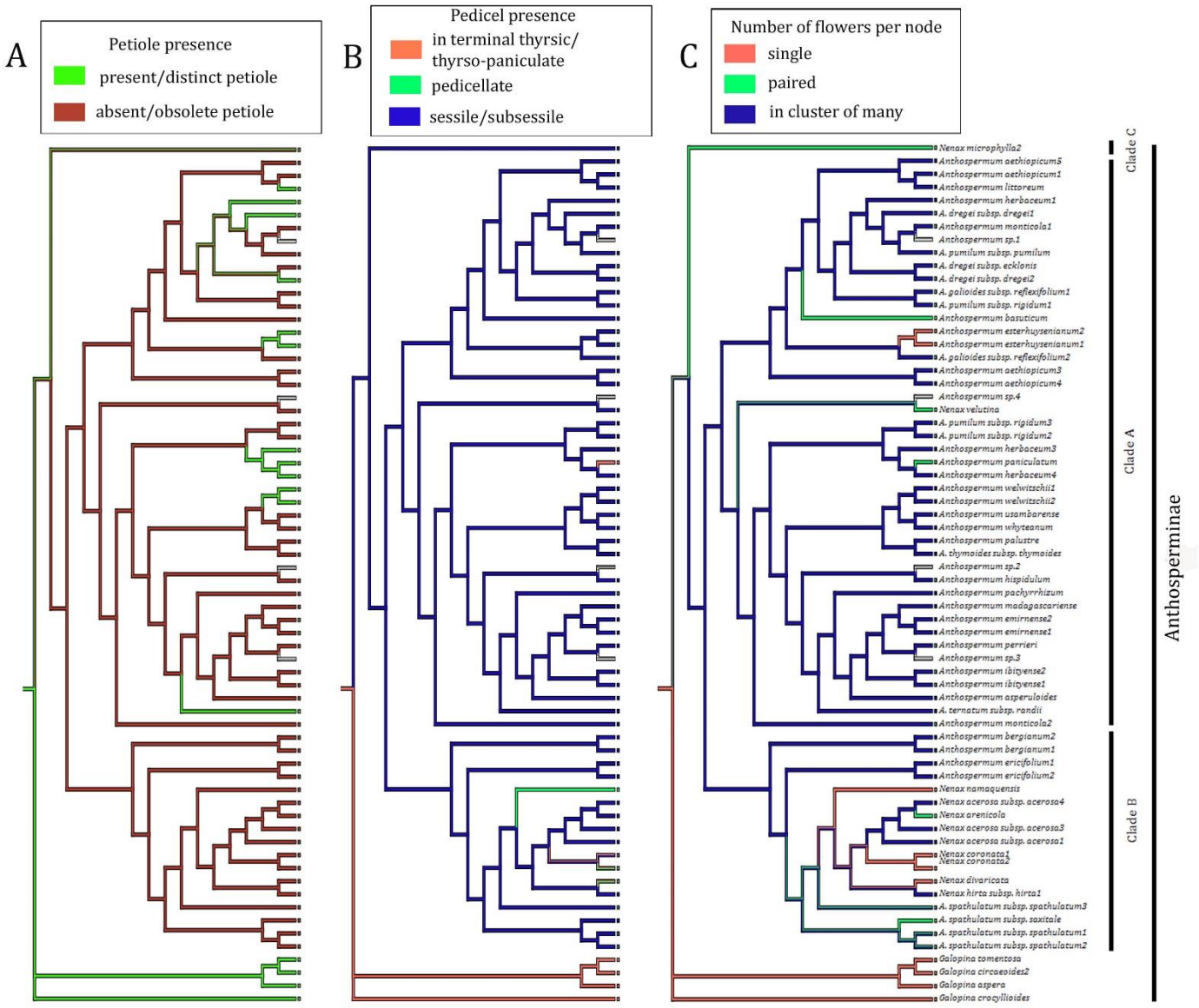

FIGURE 3.13. Character reconstruction of petiole presence (A), presence of pedicel (B) and the number of flowers per node (C) in the subtribe Anthosperminae, mapped on the Maximum Clade Credibility tree, produced from the combined dataset.

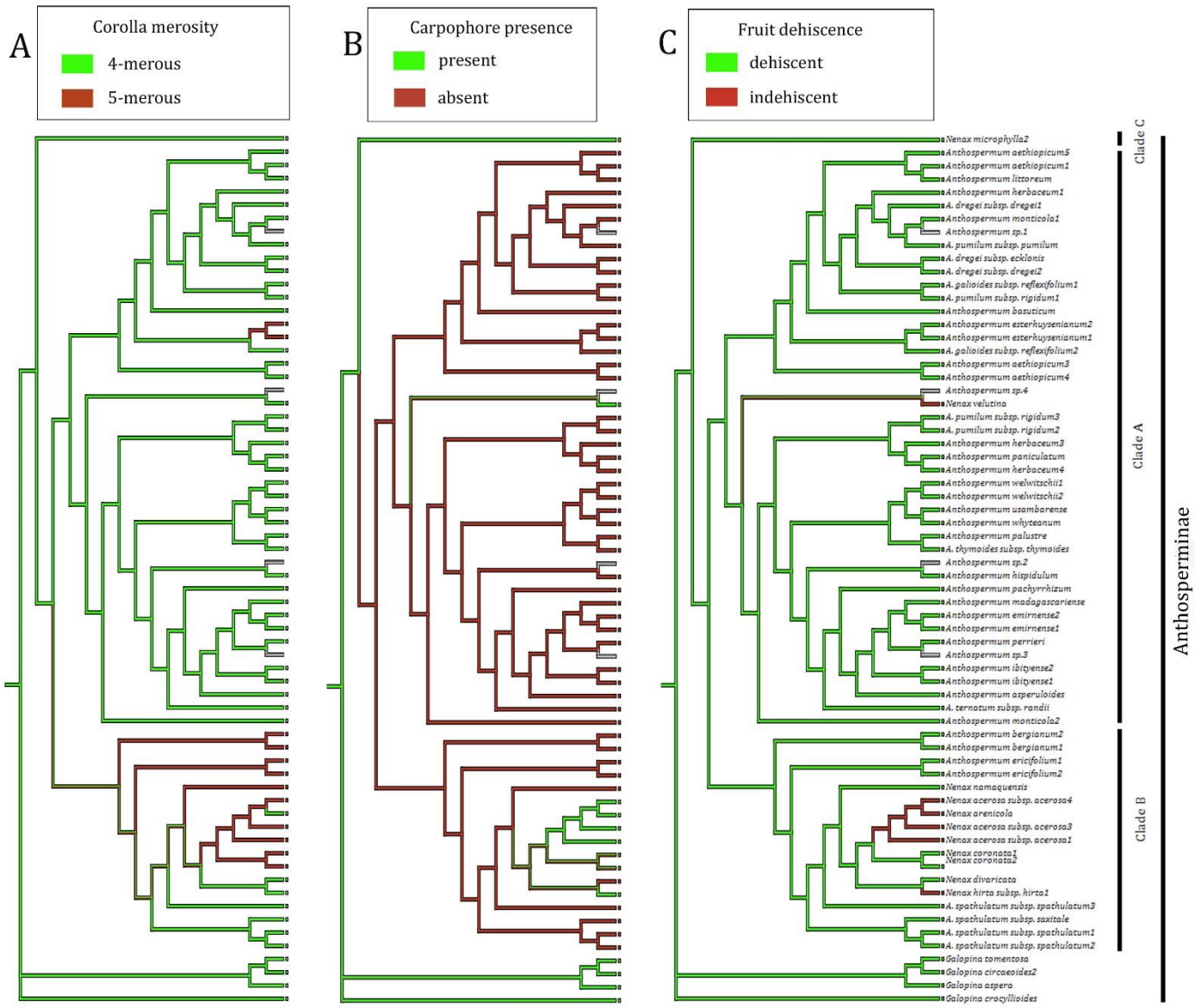

FIGURE 3.14. Character reconstruction of corolla merosity (A), carpophore presence (B) and fruit dehiscence (C) in the subtribe Anthosperminae, mapped on Maximum Clade Credibility tree, produced from the combined dataset.

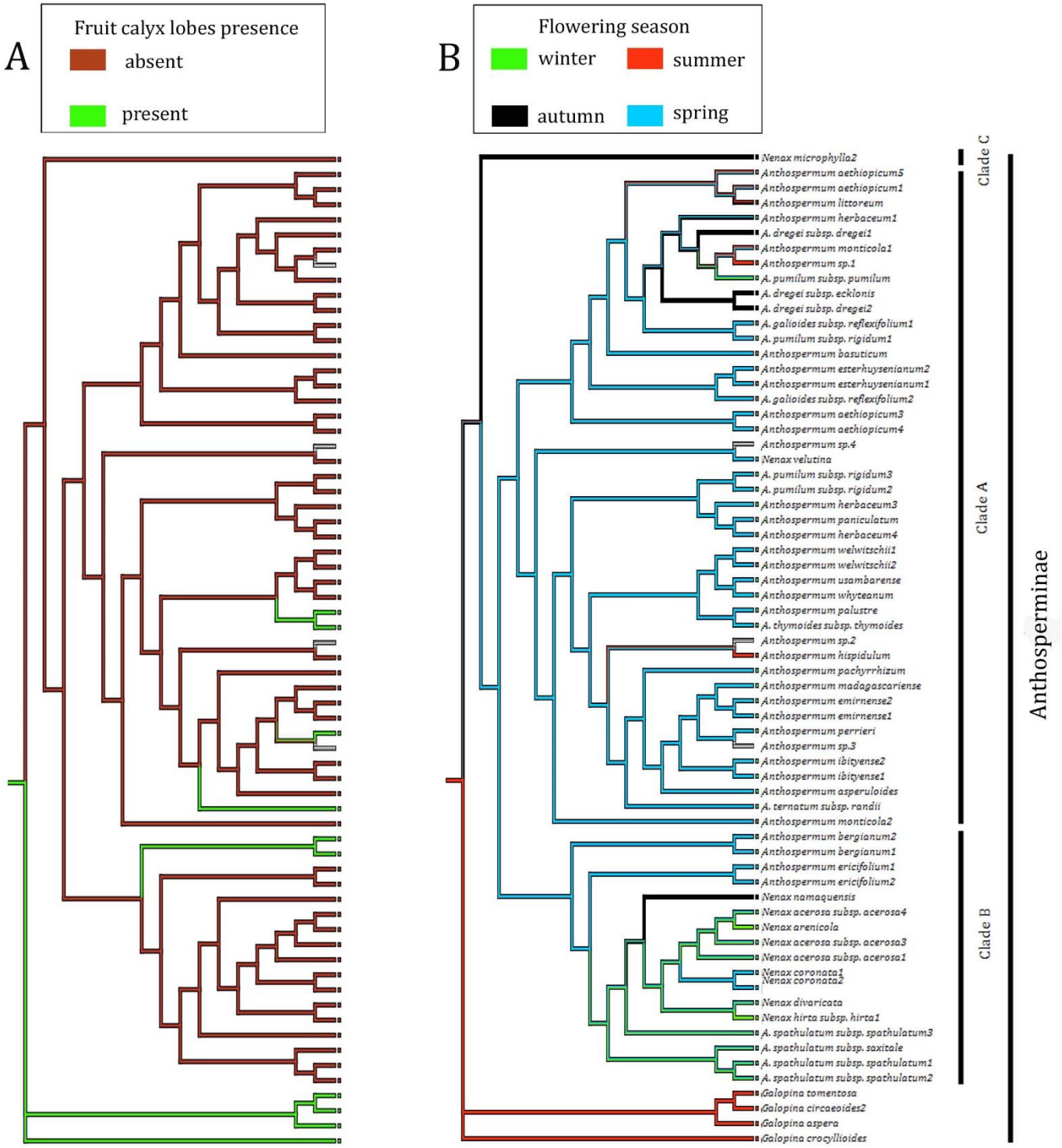

FIGURE 3.15. Character reconstruction of fruit calyx lobes presence (A), and flowering season (B) in the subtribe Anthosperminae, mapped on the Maximum Clade Credibility tree, produced from the combined dataset.

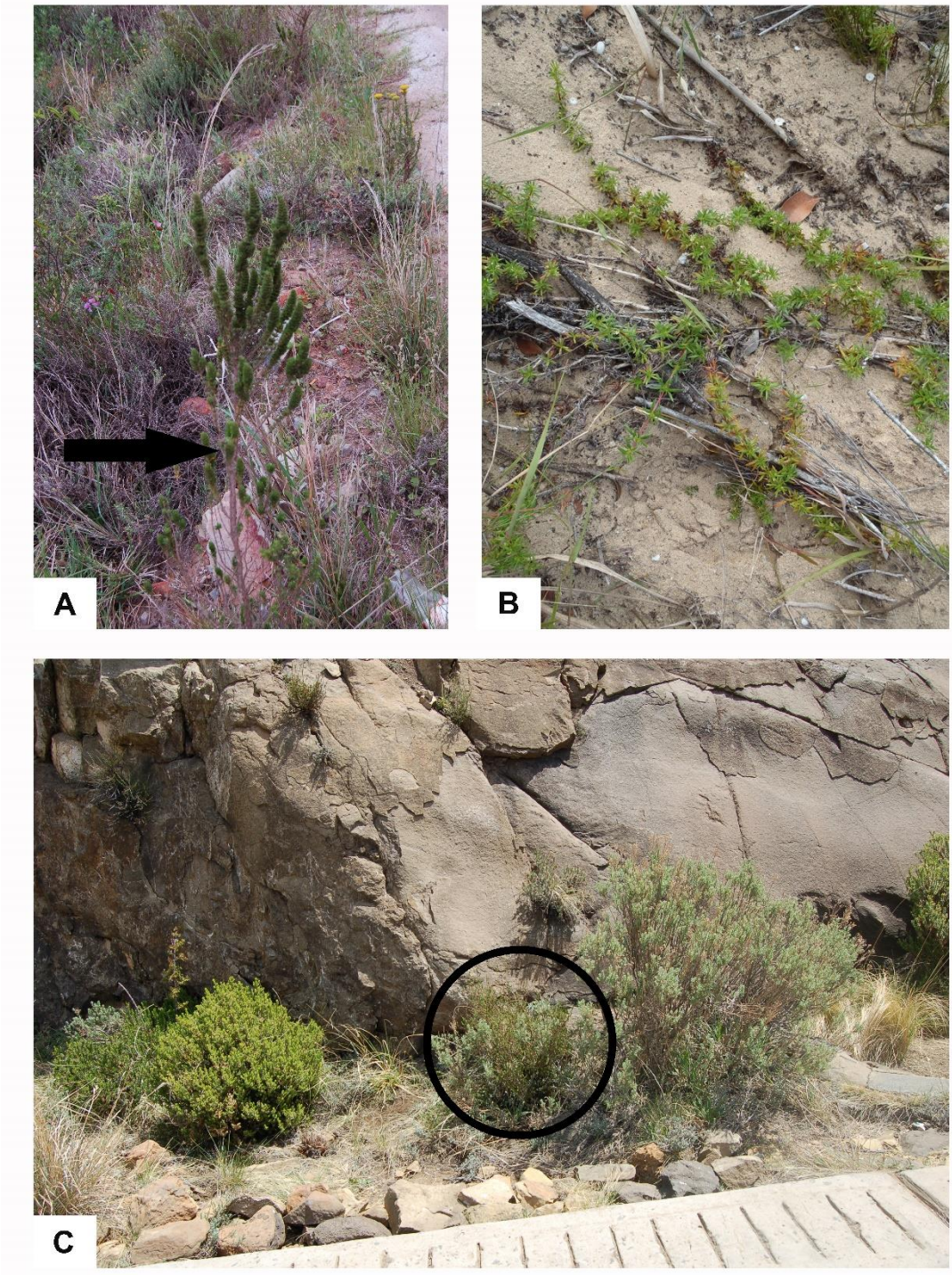

FIGURE 3.16. Growth forms of *Anthospermum*: A. Single-stemmed shrub (*A. aethiopicum*), B. Trailing subshrub (*A. prostratum*), C. Subshrub (*A. monticola*).

3.4 DISCUSSION

Extensive taxon sampling and additional markers often improve the phylogenetic signal in phylogenetic analyses, providing comprehensive results from which to assess generic circumscriptions (Heath et al. 2008). This was observed in the study by Thureborn et al. (2019) and in the present study, where expanded sampling of the subtribe Anthosperminae were included. The phylogenetic analyses presented here have provided insights into the relationships within Anthosperminae and especially the relationship between *Anthospermum* and *Nenax*.

To investigate the monophyly of the subtribe Anthosperminae, several outgroup taxa from different tribes and subtribes were selected to represent the lineages related to Anthosperminae. Within the tribe Anthospermeae, minor cases of topological conflicts were recovered, which included the position of the monotypic genus *Normandia*, *Leptostigma*, and several taxa in the *Anthospermum* - *Nenax* clade. The results of the present study were similar to those of Thureborn et al. (2019). The genus *Carpacoce* was resolved as sister to all the remaining genera of the subtribes viz. Anthosperminae, Coprosminae and Opercullariinae in all the analyses (Figs. 3.1- 3.9). The subtribe Coprosminae and Opercullariinae were both resolved in a strongly supported clade with all the genera traditionally included in these subtribes (Coprosminae: *Coprosma*, *Durringtonia*, *Nertera*, *Normandia*; and Opercullariinae: *Opercularia* and *Pomax*). The Anthosperminae clade included the genera traditionally included by Puff (1982) in the subtribe Anthosperminae, except for the genus *Carpacoce*. Therefore, our results were inconsistent with the traditional subtribal delimitation of Anthospermeae (Anthosperminae, Coprosminae, and Opercullariinae; Puff 1982), and were consistent with the recent subtribal delimitation of

Anthospermeae (Anthosperminae, Coprosminae, Carpacocinae, and Opercularrinae; Thureborn et al. 2019).

With the genus *Carpacoce* excluded from the traditional delimitation of Anthosperminae (Thureborn et al. 2019), the remaining genera i.e. the African genera *Anthospermum*, *Galopina*, *Nenax* and Macaronesian genus *Phyllis*, are now referred to as Anthosperminae s.s., and are strongly supported as a monophyletic clade in all the analyses in this study as well as previous studies (Anderson et al. 2001; Bremer and Eriksson 2009; Rydin et al. 2009b; Thureborn et al. 2019). The monophyly of *Phyllis*, *Galopina* and the *Anthospermum-Nenax* clade is consistently strongly supported, with *Phyllis* and *Galopina* occupying positions outside the *Anthospermum-Nenax* clade. However, the relationship between *Phyllis* and *Galopina* is uncertain in the Maximum likelihood analysis (Fig. 3.9) and other analyses (plastid and nuclear). Morphologically, both *Phyllis* and *Galopina* share similar inflorescence and leaf characteristics (Thureborn et al. 2019).

The two large, often single-stemmed shrubby species (*Phyllis nobla* L. and *Phyllis viscosa* Webb ex Christ.) are both included in this study. *P. nobla* is known to occur in the Canary and Madeira Islands generally in the Macaronesian laurel forest and *P. viscosa* is restricted to the Canary Islands (Thureborn et al. 2019). The genus *Galopina* is comprised of four perennial herbs, all of which were included in this study. *Galopina* species can be distinguished by their relatively broad (Fig. 3.12C) and decussately arranged leaves (Fig. 3.12A) and terminal paniculate to thyrso-paniculate inflorescence (Fig. 3.13B; Puff 1986). The species are distributed in Eswatini (Swaziland), KwaZulu-Natal and Eastern Cape, with one species (*G. circaeioides* Bär.) extending to Limpopo, Zimbabwe, Mozambique, southern Malawi, and southwest to the Cape Floristic Region

(Puff 1986; Thureborn et al. 2019). The genus *Galopina* occurs from moist and shady places to more sun-exposed places in sandy or clay soil between rocks or in grassland habitat (Puff 1986).

As mentioned before, previous molecular studies have indicated a close relationship between *Anthospermum* and *Nenax* (Anderson et al. 2001; Rydin et al. 2009b; Thureborn et al. 2019). Anderson et al. (2001) included one species from each genus and they were strongly supported as sister taxa. In the most recent study by Thureborn et al. (2019), 25 taxa of *Anthospermum* and five taxa of *Nenax* were included. In the present study with the current sampling of 29 taxa of *Anthospermum* and 10 taxa of *Nenax*, the results were in agreement with those of Thureborne et al. (2019) in which neither genus was resolved as monophyletic in all analyses. Although the topology differed between analyses, showing some taxa in different positions, three main clades within the *Anthospermum* - *Nenax* clade were recovered in all analyses, except in maximum parsimony and Bayesian inference based on the combined nuclear dataset and Bayesian inference based on the combined plastid dataset.

Nenax species were recovered in both clades, and there are no clear synapomorphies to support the distinction between *Nenax* and *Anthospermum* species, as most of the characters were shared between the genera viz. *Anthospermum* and *Nenax* share similar habits (Fig. 3.11A) in which they both are either woody shrubs or woody subshrubs, although *Anthospermum* has two species (*A. palustre* Homolle ex Puff and *A. thymoides* Baker) that are perennial herbs. The leaves decussate or arranged in whorls of three is shared between *Anthospermum* and *Nenax* species in clade A, B and C (Fig. 3.12A). The majority of *Anthospermum* and *Nenax* species share glabrous leaves with few species showing hairy leaves *A. whyteanum* Hiern., *A. hispidulum* E.Mey. ex

Harv. & Sond., *A. perrieri* Homell ex Puff, *A. bergianum* and *N. hirta* subsp. *hirta* in the different clade, (Fig. 3.12 B). *Nenax* species are often confused with *Anthospermum* species because of their shared short and thin leaves (Fig. 3.12 C), and short or obsolete petioles (Fig. 3.13 A). *Anthospermum* and *Nenax* share flowers that are subsessile or with very short pedicels (Fig. 3.13 B). *Nenax* species comprises both dehiscent and indehiscent fruits without carpophores (Fig. 3.14 B, C), while they are exclusively dehiscent with carpophores in *Anthospermum*. As mentioned earlier, *Nenax* is distinguished from *Anthospermum* through a combination of characters, woody shrub, needle-like leaves, few-flowered infloresence and dioecy. However, our results have shown an unequivocal overlap between the characters used for distinguishing the two genera.

Additionally, there are no clear synapomorphies to support the relationship between the three subclades that were recovered within the *Anthospermum - Nenax* clade (Fig. 3.7 - 3.9). However, there is support for geographical groupings in which the species are grouped according to their sampling location (Thureborn et al. 2019),i.e. clade A comprises of both species collected from Africa south of the Sahara and Madagascar, while clade B and C are comprised of species collected from southern Africa where there is the highest concentration of *Anthospermum* and *Nenax* species. With careful consideration of the molecular analyses where *Nenax* species are embedded within *Anthospermum* (Fig. 3.2 – 3.9), and combination of the following similar morphological characters: woody habit (Fig. 3.11 A), leaf arrangement (Fig. 3.12 A), leaf size (Fig. 3.12 C), short petioles (less than 0.5 mm; Fig. 3.13 A), and fruit separating into two mericarps with or without a carpophore or indehiscent without carpophore (Fig. 3.14 B and C), an expansion of the generic concept of *Anthospermum* to include *Nenax* seems most logical, and long overdue. The subtribe Anthosperminae will

now be comprised of *Anthospermum* s.l., *Galopina* and *Phyllis*. As indicated by Thureborn et al. (2019) we also keep the current delimitation of Coprosminae and Opercularrinae for future study.

CHAPTER 4: GENERIC CIRCUMSCRIPTION OF *ANTHOSPERMUM* AND *NENAX*

4.1 GENERIC CONCEPT DISCUSSION

4.1.1 Generic circumscription—As alluded in an earlier discussion broadly in chapter 3, the two genera *Anthospermum* and *Nenax* share common characteristics. This has made identification of species between the two genera more difficult, the character reconstruction analyses in this study has indicated an overlap between the currently used characters to difine the two genera**.** The new system proposed here is based on a wider consideration of the complex relationships among the two genera *Anthospermum* L. and *Nenax* Gaertn. This close relationship between *Anthospermum* and *Nenax* has also been reflected in previous studies of Puff (1986); Anderson et al. (2001); Rydin et al. (2009b); and Thureborn et al. (2019). Molecular results and morphological analyses suggest that distinguishing characters between the taxa will have to be redefined, as such the new system is proposed here with the expansion of *Anthospermum* s.s. to include the genus *Nenax*. The improvement in the generic delimitations is also reflected in the following descriptions.

Anthospermum L., Sp. Pl. 2: 1058 (1753), Gen. Pl., ed. 5: 479 (1754); Sond. in Harv. & Sond Fl. Cap. 3: 26 (1865); Hook. f. in Gen. Pl. 2(1): 140 (1873); K. Schum. in Nat. Pflanzenfam. IV, 4: 129 (1891); R.A. Dyer, Gen. S. Afr. Fl. Pl. 1: 622 (1975); Verdc. Fl. Trop. E. Africa, 1:324 (1976); Puff Fl. S. Africa 31, 1(2): 8 (1986), **emend. nov.** R.Nemando. TYPE: *A. aethiopicum* L., Sp. Pl.: 1058 (1753).

Ambraria Heist. ex Fabr., Enum. (ed. 2). [Fabr.]. 435 (1763). **syn. nov.** TYPE: *A. aethiopicum* L., Sp. Pl.: 1058 (1753). illeg. superfl. nom; non Ambraria Cruse (1825).

Nenax Gaertn., Fruct. Sem. Pl. 1. 165. t. 32 (1788); Hook. f. in Gen. Pl. 2(1): 140 (1873); K. Schum. in Nat. Pflanzenfam. IV, 4: 129 (1891); Puff in Fl. S. Africa 31, 1(2): 8 (1986). **syn. nov.** TYPE: *N. acerosa* Gaertn., Fruct. Sem. Pl. 1. 165. t. 32 (1788).

Ambraria Cruse [non Heist. ex Fabr.], Rub. Cap.: 16 (1825), in Linnaea 6: 18 (1831), Sonder in Harvey & Sonder, Fl. Cap. 3:33 (1865). **syn. nov.** TYPE: *A. glabra* Cruse. [syn of *N. acerosa* Gaertn.] Rub. Cap.: 17 (1825) illeg. superfl. nom.

Dioecious or non-dioecious large or dwarf shrubs, or short-lived subshrubs to perennial herbs (rarely), with thick woody rootstock. **Leaves** decussate or in whorls of 3 (4), often in seemingly larger number at the nodes, leafy short shoots, blades ericoid and small, mostly narrowed at base, acute to acuminate, sometimes mucronate at apex, shortly petiolate to sessile, with cup-shaped stipular bearing one to many or without satae on either side. **Inflorescence** often leafy and inconspicuous, mostly subsessile, many to few (one) flowered cymes (in some dioecious taxa often sexually dimorphic; ♀ inflorescence contracted, cylinder-like), arranged in pairs or single at nodes or flowers solitary and terminal on shoots. **Flowers** subsessile, subtended by a pair of leafy bracts, ♂, ⚥ or ♀, 4-5(-6) merous. **Calyx** lobes large to small or subobsolete. ⚥, ♂: **Corolla** tube cylindrical, broadly funnel-shaped to subcampanulate, lobes recurved, ± lanceolate. **Stamens** with anthers yellowish to whitish, exserted, dangling on long slender filiform filaments; minute rudimentary ovary usually present. ♀: corolla much smaller; tube cylindrical, lobes mostly erect, linear to ± lanceolate, **Ovary** bicarpellate and biovulate (in *A. ericifolium* and *A. bicorne* 1 carpel reduced); style 0 or very short; stigmas 2, only

A. ericifolium 1, long exserted, hairy, greyish to greenish-white, seldom purplish red. **Fruits** round in outline, occasionally hard, crowned by persistent calyx lobes, separating into two mericarps, each convex on dorsal side, with or without a U-shaped carpophore or indehiscent without carpophore. Chromosome number: 2n = 22, 44, seldom 66.

Distribution and ecology—Widely distributed across Africa south of the Sahara and in Madagascar, with the highest concentration of taxa restricted in South Africa (Puff 1986; Manning and Goldblatt 2012; Thureborn et al. 2019). One species (*A. herbaceum*) extends into the Yemen Arab Republic and adjacent parts of Saudi Arabia. Most species occur in dry to very dry habitats such as rocky outcrops, rocky slopes, and rocky grassland, gravelly to rocky flats or sandy areas (Puff 1986; Manning and Goldblatt 2012). The Afromontane species occur at the edge of the forest or in the scrub forest (Puff 1986).

4.2 REVISED KEY TO *ANTHOSPERMUM SENSU LATO*

4.2.1 Generic key for the southern African subtribe Anthosperminae (Adapted from Puff (1986))

1a. Fruit separating into exocarp-valves and endocarp plus seed; flowers mostly with only 1 fertile carpel and 1 stigma; corolla 5–7-merous, lobes distinctly hooded; calyx lobes mostly large, leaf-like ……***Carpacoce***

1b. Fruit indehiscent or separating into 2 indehiscent mericarps; flowers with 2 fertile carpels and 2 stigmas; corolla 4–5-merous, lobes not hooded; calyx lobes mostly small or subobsolete:

2a. Leaves strictly decussate, blade relatively large and thin, distinctly petiolate; perennial herbs with rhizomes or rootstocks; inflorescence terminal, paniculate to

thyrso-paniculate; fruit without calyx lobes, separating into two mericarps but lacking a carpophore..... ***Galopina***

2b. Leaves decussate or in whorls of 3, blades mostly small and narrow, frequently ericoid, often without distinct petioles; large shrubs, dwarf shrubs, short-lived subshrubs or perennial herbs; inflorescence mostly variously congested, frequently much reduced and inconspicuous; fruit often with or without small calyx lobes, separating into two mericarps with or without a carpophore or indehiscent without carpophore..... ***Anthospermum s.l.***

4.3 LIST OF RECOGNISED SPECIES

4.3.1 Recognised species in southern Africa— *A. basuticum* Puff*, A. bergianum* Cruse*, A. bicorne* Puff, *A. comptonii* Puff, *A. dregei* Sond. subsp. *dregei*, *A. dregei* subsp. *ecklonis* (Sond.) Puff, *A. eathiopicum* L., *A. ericifolium* (Licht. Ex Roem. & Schult.) Kuntze, *A. esterhuysenianum* Puff var. *esterhuysenianum, A. esterhuysenianum* var. *hirsutum* Puff, *A. galpinii* Schltr., *A. galioides* Reichb. subsp. *galioides*, *A. galioides* subsp. *reflexifolium* (Kuntze) Puff, *A. herbaceum* L., *A. hirtum* Cruse, *A. hispidulum* E. Mey. Ex Harv. & Sond., *A. littoreum* L., *A. monticola* Puff*, A. paniculatum* Cruse*, A. pumilum* Sond. subsp. *pumilum*, *A. pumilum* subsp. *rigidum* (Eckl. & Zeyh.) Puff, *A. prostratum* Sond., *A. streyi* Puff, *A. spathulatum* Spreng. subsp. *spathulatum, A. spathulatum* subsp. *uitenhagense* Puff*, A. spathulatum* subsp. *saxatile* Puff*, A. spathulatum* subsp. *ecklonianum* (Cruse) Puff*, A. spathulatum* subsp. *tulbaghense* Puff*, A. welwitschii* Hiern.

4.3.2 New combinations

1. ***Anthospermum microphylla*** (Sond.) R.Nemando, Boatwr. & Magee**, comb. nov**.

Nenax microphylla (Sond.) T. M. Salter in J. S. African Bot. 3: 113 (1937); Hobson

& Jessop, Veld Pl. S. Afr.:220, p1. 21 (1975); Puff in Fl. S. Africa 31, 1(2): 38 (1986). *Ambraria microphylla* Sond. in Harv. & Sond Fl. Cap. 3: 34 (1865). TYPE: SOUTH AFRICA, Free State, Sandrivier, *Zeyher s.n.* [*769*] (lectotype: SAM! [designated by Puff 1986]; isolectotype: BM, G [photo!], K [photo!], PRE [photo!], S [photo!]), [OTHER ORIGINAL MATERIAL] *Burke 506* (BM, K [photo!], PRE [photo!], SAM!).

2. ***Anthospermum cinerea*** (Thunb.) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax cinerea* (Thunb.) Puff in Fl. S. Africa 31, 1(2): 40 (1986). *Cliffortia cinerea* Thunb., Prodr. Pl. Cap. 2: 93 (1800). TYPE: [SOUTH AFRICA, Northern Cape], "CBS", *Thunberg (Sheet 23686)* (holotype: UPS; isotype: LU [photo!], S [photo!]).

Nenax dregei L. Bolus in Ann. S. African. Mus. 9: 215 (1917); Launert & Roessler in Merxmüller, Prod. Fl. S.W.A. 115: 19 (1966). TYPE: SOUTH AFRICA, Northern Cape, Bot Riverbed, between Calvinia and Holle River, *Pearson 3966* (lectotype: BOL! [designated by Puff 1986], isolectotype: NBG!), [OTHER ORIGINAL MATERIAL: SOUTH AFRICA] ca. 24 km North of Alewyn's Fontein (Aalwynsfontein), *Pearson 3930* (BOL!, K [photo!]); *Pearson 3295* (BOL!); SOUTH AFRICA, between Anenous and Chubiessis Outspan, *Pearson 5979* (BOL!).

Nenax hantamensis Schlechter, *nom*. *nud*. SOUTH AFRICA, Cape Province [Northern Cape], Calvinia District, Hantam Mts., *Marloth 10444* (PRE).

3. ***Anthospermum namaquensis*** (Puff) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax namaquensis* Puff in Fl. S. Africa 31, 1(2): 41 (1986). TYPE: SOUTH AFRICA, Northern Cape, Namaqualand, a little North of Middelkraal, *Pearson 5615* (holotype: BOL!, isotype K [photo!]).

4. ***Anthospermum coronata*** (Puff) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax coronata* Puff in Fl. S. Africa 31, 1(2): 41 (1986). TYPE: SOUTH AFRICA, Northern Cape, [3218 BB] West side of Pakhuis Pass, a little West of Leipoldt grave, *Puff 800902-6/4* (holotype: WU, isotype: BOL!, NBG!, PRE [photo!]).

5. ***Anthospermum divaricata*** (T.M. Salter) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax divaricata* T.M. Salter in J. S. African Bot. 3: 113 (1937); Puff in Fl. S. Africa 31, 1(2): 43 (1986). *Ambraria acerosa* Sond. in Harv. & Sond Fl. Cap. 3: 34 (1865), *pro parte*. TYPE: SOUTH AFRICA, Western Cape, Worcester District, near Tulbaghskloof, *Ecklon & Zeyher 2319 (or "1.9")* (holotype: SAM!, isotype: E [photo!], GOET [photo!], M [photo!], MO [photo!], NBG!, PRE [photo!], S [photo!], US [photo!], W [photo!], WU [photo!]).

Nenax acerosa sensu Ecklon & Zeyher, Enum. Comm. Pl. Afr. Austr. (Meyer) 1: 368 (1836), non Gaertn.

6. ***Anthospermum elsieae*** (Puff) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax elsieae* Puff in Fl. S. Africa 31, 1(2): 43 (1986). TYPE: SOUTH AFRICA, Western Cape, [3319 BD] Worcester District, Bonteberg, Eikenbosch Hoek, *Esterhuysen 3656* (holotype: BOL!).

7. ***Anthospermum hirta*** (Cruse) R.Nemando, Boatwr. & Magee, **comb. nov.**

Two subspecies are recognised:

a. ***Anthospermum hirta*** (Cruse) R.Nemando, Boatwr. & Magee subsp. ***hirta***. *Nenax hirta* (Cruse) T.M. Salter in J. S. African Bot. 3: 113 (1937), in Adamson & Salter, Fl. Cape Penins.: 735 (1950); Puff in Fl. S. Africa 31, 1(2): 44 (1986). *Ambraria hirta* Cruse, Rub. Cap.: 17 (1825), in Linnaea 6: 19 (1831); Sond. in Harv. & Sond

Fl. Cap. 3: 34 (1865). TYPE: SOUTH AFRICA, Western Cape. "C.B.S.", *Mund(T) & Maire s.n.* (syntype: B †), at base of Lion's Mt. towards Drieanckerbay, *Bergius s.n.* (syntype: B †), slopes of Signal Hill above Three Anchor Bay, *Salter 6407* (neotype and topotype: BOL!).

b. ***Anthospermum hirta*** (Puff) subsp. ***calciphila*** (Puff) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax hirta* subsp. *calciphila* Puff in Fl. S. Africa 31, 1(2): 44 (1986). TYPE: SOUTH AFRICA, Western Cape, Langebaan Peninsula, Oude Post Private Nature Reserve, *Boucher 2964* (holotype: NBG!, isotype: PRE [photo!]).

8. ***Anthospermum acerosa*** (Gaertn) R.Nemando, Boatwr. & Magee, **comb. nov.**

Two subspecies are recognised:

a. ***Anthospermum acerosa*** (Gaertn.) R.Nemando, Boatwr. & Magee subsp. ***acerosa***. *Nenax acerosa* Gaertn., Fruct. Sem. PI. 1: 165, t. 32, f. 7 (1788); T.M. Salter in J. S. African Bot. 3: 112 (1937), in Adamson & Salter, Fl. Cape Pennis.: 734 (1950); Puff in Fl. S. Africa 31, 1(2): 46 (1986). TYPE: SOUTH AFRICA, [Western Cape] South West Cape Province "C.B.S", *Masson s.n.* in herb. Banks (holotype: BM).

Cliffortia acerosa ms. [in herb. *Banks* (BM)].

Ambraria glabra Cruse, Rub. Cap.: 17 (1825), in Linnaea 6: 18 (1831); Sond. in Harv. & Sond Fl. Cap. 3: 33 (1865). *Nenax glabra* (Cruse) O. Kuntze, Rev. Gen. 31: 121 (1898). TYPE: SOUTH AFRICA, [Western Cape] South West Cape Province, "CBS", *Bergius s.n.* (holotype: B †).

Ambraria glabra var. *papillata* Sond. in Harv. & Sond Fl. Cap. 3: 34 (1865). TYPE: none cited, but almost certainly "Capfäche", 1841, *Ecklon s.n.* (holotype: S [photo!]).

Ambraria glabra Cruse var. *tulbaghica* Sond. in Harv. & Sond Fl. Cap. 3: 34 (1865). TYPE: SOUTH AFRICA, [Western Cape] Cape Province, (near) waterfall, Tulbagh, *Pappe s.n.* (holotype: S [photo!]).

Ambraria acerosa Sond. in Harv. Sond Fl. Cap. 3: 34 (1865), *pro parte*.

b. ***Anthospermum acerosa*** subsp. ***macrocarpa*** (Ecklon & Zeyher) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax acerosa* subsp. *macrocarpa* (Ecklon & Zeyher) Puff in Fl. S. Africa 31, 1(2): 46 (1986). *Ambraria hirta* var. *macrocarpa* Ecklon & Zeyher, Enum. Pl. Afr. Austr.: 368 (1836). TYPE: SOUTH AFRICA, Western Cape, on the Breederivier, Swellendam, *Mundt s.n. [Ecklon & Zeyher 2318β]* (holotype: SAM!, isotype: S [photo!]).

9. ***Anthospermum arenicola*** (Puff) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax arenicola* Puff in Fl. S. Africa 31, 1(2): 47 (1986). TYPE: SOUTH AFRICA, Western Cape, [3218 BA] Clanwilliam, ca. 3 km South East of Graafwater-Lambert's Bay rd., on Leipoldtville rd., *Puff 800915-2/1* (holotype: WU, isotype: BOL!, NBG!, PRE [photo!]).

10. ***Anthospermum velutina*** (J.C. Manning & Goldblatt) R.Nemando, Boatwr. & Magee, **comb. nov.** *Nenax velutina* J.C. Manning & Goldblatt in Strelitzia 29: 815 (2012). TYPE: SOUTH AFRICA, Western Cape, [3320 AB] Laingsburg District, [Farm] Cabidu, 28 October 1950, *Compton 22209* (holotype: NBG!, syntype: NBG!).

4.3.3 Electronic key to Anthospermum species—As indicated (in Chapter 2) a digital key to the species of *Anthospermum sensu lato* in southern Africa was produced using the DELTA software package (Dallwitz et al. 1993). This key provides a user-friendly tool for the identification of *Anthospermum* species. The genus now includes 49

species globally and due to the diverse range of growth forms and floral morphologies of the species, the distinction between species is often difficult, making identification of the species more challenging. The key offers images that significantly aid in the identification of species, as well as images illustrating diagnostic characters. A digital key is useful, as the user can select the order in which to use the characters for identification, rather than following a predetermined order. The key provides species distribution maps adapted from Puff (1986) which are also helpful in the identification of the species. https://ranganin7.wixsite.com/anthospermum

CHAPTER 5: GENERAL CONCLUSIONS

The results presented in this study have provided insights into relationships within the Anthospermeae and among the closely allied genera *Anthospermum* L. and *Nenax* Gaertn. Following the assessment of generic circumscriptions in the Anthosperminae using molecular and morphological data, the number of genera recognized was decreased from four to three.

The monophyly of the subtribe Anthosperminae was confirmed with strong support in all analyses. *Phyllis* L. and *Galopina* Thunb. were consistently recovered outside of the *Anthospermum* - *Nenax* clade in all the analyses.

The paraphyly of *Anthospermum* was confirmed, with 29 taxa, together with 10 taxa of the polyphyletic *Nenax*, both placed in the monophyletic *Anthospermum* - *Nenax* clade. The expanded phylogenetic sampling recovered the species of *Nenax* embedded within *Anthospermum*, and *Anthospermum* was subsequently expanded to include the species of *Nenax* based on shared growth forms, floral structure, fruit characteristics, and phyllotaxis (Puff 1986; Thureborn et al. 2019). The reconstruction of selected morphological characters on the phylogeny to identify phylogenetically informative characters revealed no characters that could be used to define phylogenetic groups within the *Anthospermum* - *Nenax* clade. However, the majority of species were grouped according to their sampling locality (geographically structured).

Identification of the species within the *Anthospermum* - *Nenax* clade is considered difficult, therefore, a user-friendly digital DELTA key was produced for southern Africa *Anthospermum* (including *Nenax*) species, and the key provides images illustrating diagnostic characters and distribution maps adapted from Puff (1986). The

key is useful for taxonomists, conservation agencies in the identification of species and assessment of species distribution.

Biogeographic patterns of plant lineages remain poorly understudied but are slowly beginning to emerge from recent studies (Martín-Bravo and Escudero 2012; Cantley et al. 2016), therefore, future studies on the genus *Anthospermum* and other genera within subtribe Anthosperminae should focus on biogeographic patterns, improving the resolution and strengthening the 'backbone' of the tree.

Table 4. Summary of recent generic changes within the subtribe Anthosperminae compared to the traditional classification by Puff 1982, 1986. Genera altered in the present study are indicated in bold.

Puff 1982, 1986		**Thureborn et al. 2019**		**Current study**	
GENUS	SUBTRIBE	GENUS	SUBTRIBE	GENUS	SUBTRIBE
Anthospermum **Nenax** *Galopina* *Carpacoce* *Phyllis*	Anthosperminae	***Anthospermum*** **Nenax** *Galopina* *Phyllis*	Anthosperminae	***Anthospermum*** **s.l. (including** ***Nenax*****)** *Galopina* *Phyllis*	Anthosperminae
		Carpacoce	Carpacocinae	*Carpacoce*	Carpacocinae

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APPENDIX

APPENDIX 1. Voucher information for the taxa sampled for this study. GenBank accession numbers are also given for sequences obtained from other studies. The information is listed as follows: ***Taxon***, voucher, locality, lab identification, GenBank accessions (XXXXX to be submitted): *trnl-F*, *rps16*, *rpl32*, ITS, ETS. Those regions not sampled for a taxon are represented by an em dash [([1]Andersson and Rova 1999); ([2]Anderson et al. 2001); ([3]Backlund et al. 2007); ([4]Ferm et al. 2016); ([5]Ginter et al. 2015); ([6]Groeninckx et al. 2009); ([7]Hiiesalu et al. 2012); ([8]Janssens et al. 2016); ([9]Kårehed and Bremer 2007); ([10]Kårehed et al. 2008); ([11]Krüger et al. 2012); ([12]Kool et al. 2012); ([13]Nie at al. 2013); ([14]Rydin et al. 2008); ([15]Rydin et al. 2009b); ([16]Roeder 2013); ([17]Refulio-Rodriguez and Olmstead 2014); ([18]Struwe et al. 1998); ([19]Soza and Olmstead 2010b); ([20]Shepherd et al. 2013); ([21]Thureborn et al. 2019); ([22]Wikström et al. 2010); ([23]Yang 2016); ([24]Yang et al. 2018)].

Anthospermeae: ***Anthospermum aethiopicum*** L. 1, *Nemando 41* (NBG), South Africa: Eastern Cape, RN21, XXXXX, XXXXX, XXXXX, XXXXX, —. ***Anthospermum aethiopicum*** L. 2, *Dahstrand 416* (GB), South Africa,—,—, AF257897[2], —, AF257896[2],—. ***Anthospermum aethiopicum*** L. 3, *Bremer et al. 4363* (UPS), South Africa: Western Cape, aL78, MK141633[21], MK141546[21], —, MK141274[21], MK141184[21]. ***Anthospermum aethiopicum*** L. 4, *Bremer et al. 4367* (UPS), South Africa: Western Cape, aL79, MK141634[21], MK141547[21], —, MK141275[21], MK141185[21]. ***Anthospermum aethiopicum*** L. 5, *Nemando et al. 44* (NBG), South Africa: Eastern Cape, RN25, XXXXX,

XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum asperuloides*** Hook.f., *Breteler et al. 108* (UPS), Cameroon: South-West, cX89, MK141605[21], MK141519[21], —, MK141245[21], MK141156[21]. ***Anthospermum bergianum*** Cruse. 1, *Husted LBH119* (NBG), South Africa: Western Cape, RN32, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum bergianum*** Cruse. 2, *Bremer et al. 4413* (UPS), South Africa: Western Cape, aL49, MK141521[21], MK141429[21], —, MK141158[21], MK141606[21]. ***Anthospermum basuticum*** Puff., *Hilliard & Burtt 7121* (S), South Africa: KwaZulu-Natal, cX48, —, MK141520[21], —, MK141246[21], MK141157[21]. ***Anthospermum dregei* subsp*. dregei*** Sond. 1, *Nemando 5* (NBG), South Africa: Western Cape, RN47, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum dregei* subsp*. dregei*** Sond. 2, *Acocks 15194* (S), South Africa: Western Cape, cX51, MK141609[21], MK141524[21], —, MK141250[21], MK141161[21]. ***Anthospermum dregei* subsp*. ecklonis*** (Sond) Puff., *Nemando 14* (NBG), South Africa: Western Cape, RN49, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum ericifolium*** (Licht. ex Roem. & Schult.) Kuntze. 1, *Helme 5767* (NBG), South Africa: Western Cape, RN33, XXXXX, XXXXX, XXXXX, XXXXX, —. ***Anthospermum ericifolium*** (Licht. ex Roem. & Schult.) Kuntze. 2, *Esterhuysen 35553*(S), South Africa: Western Cape, cX53, MK141611[21], MK141525[21], —, MK141252[21], MK141163[21]. ***Anthospermum esterhuysenianum* var*. esterhuysenianum*** Puff. 1, *Helme 5753* (NBG), South Africa: Western Cape, RN39, —, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum esterhuysenianum* var*. esterhuysenianum*** Puff. 2, *Esterhuysen 34159a* (S), South Africa: Western Cape, cX54, MK141612[21], MK141526[21], —, MK141253[21], —. ***Anthospermum emirnense*** Baker. 1, *Razafimandimbison & Krüger 863* (S), Madagascar: Fianarantsoa, cX68, MK141626[21], MK141539[21], —, MK141267[21], MK141177[21]. ***Anthospermum emirnense*** Baker. 2, *Razafimandimbison & Krüger 862* (S), Madagascar: Fianarantsoa, cX69, MK141627[21], MK141540[21], —, MK141268[21], MK141178[21].

Anthospermum galioides* subsp*. reflexifolium (Kuntze) Puff. 1, *Nemando 47* (NBG), South Africa: Eastern Cape, RN26, XXXXX, XXXXX, XXXXX, XXXXX, —. ***Anthospermum galioides* subsp*. reflexifolium*** (Kuntze) Puff. 2, *Bremer et al. 4379* (UPS), South Africa: Western Cape, aL82, MK141637[21], MK141550[21], —, MK141278[21], —. ***Anthospermum herbaceum*** L.F. 1, *Nemando et al. 31* (NBG), South Africa: KwaZulu-Natal, RN18, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum herbaceum*** L.F. 2, *Bremer 3093* (UPS), Tanzania, —, EU145544[14], —, JQ729926[14], EU145355[14], —. ***Anthospermum herbaceum*** L.F. 3, *Bremer et al. 4340* (UPS), South Africa: Limpopo, aL63, MK141631[21], MK141544[21], —, MK141272[21], MK141182[21]. ***Anthospermum herbaceum*** L.F. 4, *Friis & Demissew 10140* (UPS), Ethiopia: Tigray, cX90, MK141613[21], —, —, MK141254[21], MK141164[21]. ***Anthospermum hispidulum*** E.Mey. ex Sond., *Stray 7505* (S), South Africa: KwaZulu-Natal, cX57, MK141614[21], MK141528[21], —, MK141255[21], MK141165[21]. ***Anthospermum ibityense*** Puff. 1, *Eriksson & Lundberg T975* (S), Madagascar: Antananarivo, cX65, MK141624[21], MK141537[21], —, MK141265[21], MK141175[21]. ***Anthospermum ibityense*** Puff. 2, *Schatz et al. 4094* (P), Madagascar: Antananarivo, cY70, MK141615[21], MK141529[21], —, MK141256[21], MK141166[21]. ***Anthospermum littoreum*** L., *Nemando et al. 33* (NBG) South Africa: Eastern Cape, RN19, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum monticola*** Puff. 1, *Nemando et al. 18* (NBG) South Africa: Eastern Cape, RN15, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum monticola*** Puff. 2, *Hilliard & Burtt 17995* (S), South Africa: KwaZulu-Natal, cX60, MK141617[21], MK141531[21], —, MK141258[21], MK141168[21]. ***Anthospermum madagascariense*** Homolle ex Puff., *Razafimandimbison 559* (S), Madagascar: Antananarivo, cX59, MK141616[21], MK141530[21], —, MK141257[21], MK141167[21]. ***Anthospermum pachyrrhizum*** Hiern., *Ryding 2045* (UPS), Eritrea: Anseba, cX93, MK141618[21], MK141532[21], —, MK141259[21], MK141169[21]. ***Anthospermum palustre***

Homolle ex Puff., *Eriksson et al. T973* (S), Madagascar: Fianarantsoa, cX66, MK141625[21], MK141538[21], —, MK141266[21], MK141176[21]. ***Anthospermum paniculatum*** Cruse., *Wall s.n.* (S), South Africa: Eastern Cape, cX61, MK141620[21], MK141534[21], —, MK141261[21], MK141171[21]. ***Anthospermum perrieri*** Homolle ex Puff., *Rasoarivelo s.n* (P), Madagascar: Antananarivo, cY74, MK141621[21], —, —, MK141262[21], MK141172[21]. ***Anthospermum pumilum* subsp*. pumilum*** Sond., *Nemando et al. 20* (NBG), South Africa: Eastern Cape, RN16, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum pumilum* subsp*. regidum*** Eckl. & Zeyh. 1, *Nemando et al. 34* (NBG), South Africa: Eastern Cape, RN20, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum pumilum* subsp*. rigidum*** Eckl. & Zeyh. 2, *Bremer et al. 4267* (UPS), South Africa, Western Cape, aL48, MK141630[21], MK141543[21], —, MK141271[21], MK141181[21]. ***Anthospermum pumilum* subsp*. rigidum*** Eckl. & Zeyh. 3, *Wanntorp & Wanntorp 196* (S), Namibia: Khomas, cX63, MK141622[21], MK141535[21], —, MK141263[21], MK141173[21]. ***Anthospermum sp***. 1, *Boatwright 744* (NBG), South Africa: Western Cape, RN06, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum sp***. 2, *Bremer et al. 4351* (UPS), South Africa: Limpopo, aL84, MK141641[21], MK141555[21], —, MK141283[21], MK141192[21]. ***Anthospermum sp***. 3, *Bremer et al. 5336* (S), Madagascar: Antananarivo, cX67, MK141623[21], MK141536[21], —, MK141264[21], MK141174[21]. ***Anthospermum sp.*** 4, *Phillipson 4487* (P), Lesotho, cY69, MK141604[21], MK141518[21], —, MK141244[21], MK141155[21]. ***Anthospermum spathulatum* subsp*. spathulatum*** Spreng. 1, *Boucher 7001* (NBG), South Africa: Western Cape, RN28, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Anthospermum spathulatum* subsp*. spathulatum*** Spreng. 2, *Bremer et al. 4372* (UPS), South Africa: Western Cape, aL80, MK141635[21], MK141548[21], —, MK141276[21], MK141186[21]. ***Anthospermum spathulatum* subsp*. spathulatum*** Spreng. 3, *Nemando 12* (NBG), South Africa: Western Cape, RN48, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX.

Anthospermum spathulatum* subsp*. saxitale Puff., *Helme 1948* (NBG), South Africa: Western Cape, RN29, XXXXX, XXXXX, XXXXX, —, XXXXX. ***Anthospermum ternatum* subsp*. randii*** (S.Moore) Puff., *Iversen & Martinsson 89147* (UPS), Malawi: Central, cX94, MK141639[21], MK141552[21], —, MK141280[21], MK141189[21]. ***Anthospermum thymoides* subsp*. thymoides*** Baker, J., *Krüger & Razafimandimbison 70* (S), Madagascar: Fianarantsoa, cX70, MK141628[21], MK141541[21], —, MK141269[21], MK141179[21]. ***Anthospermum usambarense*** K.Schum., *Mwangoka et al. 1180* (S), Tanzania: Kilimanjaro, cX73, MK141640[21], MK141553[21], —, MK141281[21], MK141190[21]. ***Anthospermum welwitschii*** Hiern. 1, *Luke et al. 8928* (UPS), Kenya: Rift Valley, ai76, DQ662220[2], MK141554[21], —, MK141282[21], MK141191[21]. ***Anthospermum welwitschii*** Hiern. 2, *Thulin & Mhoro 3244* (UPS), Tanzania: Iringa, cX95, MK141642[21], MK141556[21], —, MK141284[21], MK141193[21]. ***Anthospermum whyteanum*** Hiern., *Brummitt 10056* (UPS), Malawi: Central, cX96, MK141643[21], MK141557[21], —, MK141285[21], MK141194[21]. ***Carpacoce spermacocea*** (Rchb. ex Spreng.) Sond. 1, *Bremer & al. 4385* (UPS), South Africa, Western Cape, aL86, FJ695404[15], FJ695261[15], —, FJ695438[15], MK141197[21]. ***Carpacoce spermacocea*** (Rchb. ex Spreng.) Sond. 2, *Helme 2873* (NBG), South Africa: Western Cape, RN09, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Carpacoce spermacocea*** (Rchb. ex Spreng.) Sond. 3, *Bremer & Bremer 3708* (UPS), South Africa: Western Cape, s44, MK141646[21], MK141560[21], —, MK141288[21], MK141198[21]. ***Carpacoce gigantea*** Puff., *Mc Donald & Marle 1055a* (NBG), South Africa: Western Cape, RN10, —, XXXXX, —, —, XXXXX. ***Carpacoce vaginellata*** Salter., *Acocks 22841* (S), South Africa: Western Cape, cX76, MK141648[21], MK141562[21], —, MK141290[21], MK141200[21]. ***Carpacoce sp***. 1, *Jardine 1678* (NBG), South Africa: Western Cape, RN34, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Coprosma foetidissima*** J.R.Forst. & G.Forst., *Skottsberg s.n.* (S), New Zealand: South Island, cY8, MK141650[21], MK141564[21], —, MK141292[21], MK141202[21]. ***Coprosma***

grandifolia Hook.f., *Skottsberg s.n.* (S), New Zealand: South Island, cY95, MK141654[21], MK141568[21], —, MK141296[21], MK141206[21]. ***Coprosma hirtella*** Labill., *Nordenstam & Anderberg s.n.* (S), Australia: Victoria, cY96, MK141655[21], MK141569[21], —, MK141297[21], MK141207[21]. ***Coprosma lucida*** J.R.Forst. & G.Forst., *Tibell NZ210* (UPS), New Zealand: South Island, cY9, MK141657[21], MK141571[21], —, MK141299[21], MK141209[21]. ***Coprosma montana*** Hillebr., *Degener & Degener 34421* (UPS), United States: Hawaii, cY98, MK141658[21], MK141572[21], —, MK141300[21], MK141210[21]. ***Durringtonia paludosa*** R.J.F.Hend. & Guymer. 1, *NSW 154507*(NSW), —, —, AF257917[2], —, AF257916[2], —. ***Durringtonia paludosa*** R.J.F.Hend. & Guymer. 2, *Henderson et al. H3044* (NSW), Australia: New South Wales, bv75, MK141662[21], MK141576[21], —, MK141304[21], MK141214[21]. ***Durringtonia paludosa*** R.J.F.Hend. & Guymer. 3, *Henderson et al H3048* (P), Australia: New South Wales, cY80, MK141663[21], MK141577[21], —, MK141305[21], MK141215[21]. ***Galopina aspera*** (Eckl. & Zeyh.) Walp., *Phillipson 1461* (UPS), South Africa: Eastern Cape, cX98, MK141664[21], MK141578[21], —, MK141306[21], MK141216[21]. ***Galopina circaeoides*** Thunb. 1, *Golablat 6779* (NBG), —, RN12, —, —, —, XXXXX, —. ***Galopina circaeoides*** Thunb. 2, *Bremer & Bremer 3797* (UPS), South Africa: Mpumalanga, cY6, MK141665[21], MK141579[21], —, —, MK141217[21]. ***Galopina crocyllioides*** Bär., *Hilliard & Burtt 10184* (P), South Africa: KwaZulu-Natal, cY81, MK141666[21], MK141580[21], —, MK141307[21], MK141218[21]. ***Galopina tomentosa*** Hochst., *Stray 9561* (S), South Africa: KwaZulu-Natal, cX80, MK141667[21], MK141581[21], —, MK141308[21], MK141219[21]. ***Leptostigma pilosum*** (Benth.) Fosberg. 1, *Molau & Eriksen 2193* (GB), —, —, —, AF002739[1], —, AF257919[1], —. ***Leptostigma pilosum*** (Benth.) Fosberg. 2, *Erik Asplund 7171* (UPS), Ecuador: Imbabura, cX99, —, —, —, MK141309[21], MK141220[21]. ***Leptostigma reptans*** (F.Muell.) Fosberg., *NSW 276692* (NSW), —, —, —, AF257921[2], —, AF257920[2], —. ***Nenax acerosa* subsp*. acerosa***

Gaertn. 1, *Boucher 7509* (NBG), South Africa: Western Cape, RN44, XXXXX, XXXXX, XXXXX, —, XXXXX. ***Nenax acerosa* subsp*. acerosa*** Gaertn. 2, *Esterhuysen 33327* (S), South Africa: Western Cape, cY61, MK141668[21], MK141582[21], —, —, —. ***Nenax acerosa* subsp*. acerosa*** Gaertn. 3, *Hafström & Acock 1432* (S), South Africa: Western Cape, cY62, MK141669[21], MK141583[21], —, MK141311[21], MK141221[21]. ***Nenax acerosa* subsp*. acerosa*** Gaertn. 4, *Boucher 7478* (NBG), South Africa: Western Cape, RN38, XXXXX, XXXXX, XXXXX, XXXXX, —. ***Nenax arenicola*** Puff., *Acocks 19635* (UPS), South Africa: Western Cape, cX100, MK141670[21], MK141584[21], —, MK141312[21], MK141222[21]. ***Nenax coronata*** Puff. 1, *Nemando 10* (NBG), South Africa: Western Cape, RN51, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Nenax coronata*** Puff. 2, *Nemando 8* (NBG), South Africa: Western Cape, RN52, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Nenax cinerea*** (Thunb.) Puff., *Helme 7306* (NBG), South Africa: Western Cape, RN41, XXXXX, XXXXX, XXXXX, —, —. ***Nenax divaricata*** Salter., *Acocks 17458* (UPS), South Africa: Northern Cape, cY1, MK141671[21], MK141585[21], —, MK141313[21], MK141223[21]. ***Nenax hirta* subsp*. hirta*** (Cruse) Salter. 1, *Hafström & Acock 1433* (S) South Africa: Western Cape, cX82, MK141672[21], MK141586[21], —, MK141314[21], MK141224[21]. ***Nenax hirta* subsp*. hirta*** (Cruse) Salter. 2, *Boucher 7511* (NBG), South Africa: Western Cape, RN43, —, XXXXX, XXXXX, —, XXXXX. ***Nenax microphylla*** (Sond.) Salter. 1, *Golablat & Manning 12651* (NGB), South Africa: Northern Cape, RN40, —, —, —, XXXXX, XXXXX. ***Nenax microphylla*** (Sond.) Salter. 2, *Hafström & Acock 1441* (S), South Africa: Western Cape, cX83, MK141673[21], MK141587[21], —, MK141315[21], MK141225[21]. ***Nenax namaquensis*** Puff., *Rosch HR 786* (NBG), South Africa: Western Cape, RN42, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Nenax sp.***, *Helme 4544* (NBG), South Africa: Western Cape, RN45, —, XXXXX, XXXXX, —, —. ***Nenax velutina*** Manning J.C. & Golablat, sp. Nov., *CR17116* (NBG), South Africa: Western Cape, RN08, XXXXX, XXXXX, XXXXX, XXXXX, XXXXX. ***Nertera***

dichondrifolia (A.Cunn.) Hook.f., *Tibell NZ119* (UPS), New Zealand: West Coast, cY3, MK141674[21], MK141588[21], —, MK141316[21], MK141226[21]. ***Nertera granadensis*** (Mutis ex L.f.) Druce. 1, *Persson & Gustafsson 368* (S), Bolivia, cX77, MK141653[21], MK141567[21], —, MK141295[21], MK141205[21]. ***Nertera granadensis*** (Mutis ex L.f.) Druce. 2, *Chung & Anderberg 1348* (S), Taiwan, cX79, MK141651[21], MK141565[21], —, MK141293[21], MK141203[21]. ***Nertera holmboei*** Christoph., *Christophersen 2021* (P), Tristan da Cunha, cY82, MK141675[21], MK141589[21], —, MK141317[21], MK141227[21]. ***Normandia neocaledonica*** Hook. f. 1, *Munzinger 532* (MO), New Caledonia, af77, EU145543[14], MK141591[21], —, MK141319[21], MK141228[21]. ***Normandia neocaledonica*** Hook. f. 2, *Selling 125.b* (S), New Caledonia, cY63, MK141677[21], MK141592[21], —, MK141320[21], MK141229[21]. ***Opercularia hirsuta*** F.Muell. ex Benth., *Nordenstam & Anderberg 1989* (S), Western Australia, cX84, MK141678[21], MK141593[21], —, MK141321[21], MK141230[21]. ***Opercularia scabrida*** Schltdl., *Blaylock 2072* (S), South Australia, cX86, MK141680[21], MK141595[21], —, MK141322[21], MK141232[21]. ***Opercularia spermacocea*** Juss., *Morat 8340* (P), Western Australia, cY88, MK141681[21], MK141596[21], —, MK141323[21], MK141233[21]. ***Opercularia turpis*** F.Muell., *Jeanes & Lay 2485* (S), Australia: Victoria, cX87, MK141682[21], MK141597[21], —, MK141324[21], MK141234[21]. ***Opercularia varia*** Hook.f., *Karunajeewa 832* (S), Australia: Victoria, cY4, MK141683[21], MK141598[21], —, MK141325[21], MK141235[21]. ***Phyllis nobla*** L. 1, *Anderson 2203* (GB), —, —, —, AF003613[2], —, AF257939[2], —. ***Phyllis nobla*** L. 2, *Anderberg et al.* (S), Madeira, cY59, MK141687[21], MK141600[21], —, MK141328[21], MK141239[21]. ***Phyllis viscosa*** Webb ex Christ., *Santesson 26911* (S), Tenerife, cX88, MK141688[21], MK141601[21], —, MK141329[21], MK141240[21]. ***Pomax umbellata*** (Gaertn.) Sol. ex A.Rich. 1, *Andersson 2258* (GB), —, —, —, AF257941[2], —, AF257940[2], —. ***Pomax umbellate*** (Gaertn.) Sol. ex

A.Rich. 2, *Bremer & Bremer 3918* (UPS), Australia: New South Wales, v7, FJ695420[15], MK141602[21], —, MK141330[21], MK141241[21].

Outgroup: ***Agathisanthemum bojeri*** Klotzsch., *Dessein et al. 671* (BEL), —, —, EU543077[10], EU543018[10], —, AM939424[10], —. ***Agathisanthemum chlorophyllum*** (Hochst.) Bremek., *Galfrin m174* (A), —, —, —, HE649787[10], —, HE657657[10], HE681450[10]. ***Agathisanthemum globosum*** Klotzsch., *Dessein et al. 201* (BEL), —, —, EU543078[10], EU543019[10], —, AM939425[10], —. ***Argostemma bifolium*** Ridl., *Bremer 1797* (S), —, —, FJ695396[5], —, —, —, KP212732[5]. ***Argostemma hookeri*** King. 1, *Wanntorp 88-27* (S), —, —, FJ695400[15], FJ695255[15], —, FJ695432[15], —. ***Argostemma hookeri*** King. 2, *Wanntorp s.n.*, —, —, —, —, JQ729927[11], —, —. ***Argostemma rupestre*** Ridl. 1, *B. Bremer & K. Bremer 1675* (S), —, —, —, FJ695259[15], —, FJ695436[15], —. ***Argostemma rupestre*** Ridl. 2, *B. Bremer & K. Bremer 1609* (S), —, —, KP212865[5], —, — , —, KP212738[5]. ***Argostemma yappii*** King. 1, *B. Bremer & K. Bremer 1609* (S), —, —, FJ695403[15], FJ695260[15], —, FJ695437[15], —. ***Argostemma yappii*** King. 2, *B. Bremer & K. Bremer 1675* (S), —, —, —, —, —, —, KP212739[5]. ***Conostomium natalense*** (Hochst.) Bremek. 1, *Dahlstrand 1346* (GB), —, —, EU543085[10], —, —, AM939435[10], AM932925[10]. ***Conostomium quadrangulare*** (Rendle) Cufod., *Puff & Kelbessa 821222 2/2* (UPS), —, —, EU543086[10], EU543024[10], —, AM939436[10], AM932926[10]. ***Conostomium zoutpansbergense*** (Bremek.) Bremek., *Bremer et al. 4331* (UPS), —, —, EU543087[10], —, —, AM939437[10], AM932927[10]. ***Galium aparine*** L. 1, *Boufford et al. 38776* (KUN), —, —, —, —, —, KP098102[23], —. ***Galium aparine*** L. 2, *Fraga et al. 1282* (RSA), —, —, HQ412968[17], —, —, —, —. ***Galium aparine L.*** **var*. tenerum***, *YangLE332* (KUN), —, —, MG906475[24], —, —, MG906005[24], MG906079[24]. ***Galium album*** Mill. 1, *Hiiesalu 29*, Estonia, —, HM590289[7], —, —, —, —. ***Galium baillonii*** Brantza, —, —, —, GU357103[19], —, —, —, HM061064[19]. ***Knoxia manika*** (Verdc.) Puff & Robbr., *Schaijes 3339* (BR), —,

—, —, AM266825[13], —, AM267001[13], —. ***Knoxia platycarpa*** Arn., *Lundqvist 11302* (UPS), —, —, AM266915[9], AM266826[9], —, AM267002[9], KT792992[4]. ***Knoxia sumatrensis*** Wall., *Klackenberg & Lundin 268* (S), —, —, AM266916[9], AM266827[9], JQ729923[11], AM267003[9], —. ***Otiophora pauciflora*** Baker., *Eriksson et al. T1023* (S), —, —, FN376352[22], FN376340[22], —, FN376363[22], —. ***Paederia foetida*** L. 1, *Liana Mengsong 381_1_6* (HITBC), —, —, —, —, —, HG004853[16], —. ***Paederia foetida*** L. 2, *Nie 0221* (KUN), —, —, KC306134[13], —, —, —, —. ***Plocama pendula*** Aiton., *Andreasen 1* (UPS), —, —, DQ662162[3], FJ695276[3], —, FJ695459[3], —. ***Pentanisia microphylla*** Chiov., *Thulin et al. 9206* (UPS), —, —, AM266945[9], AM266857[9], —, AM267030[9], KT792994[4]. ***Pentanisia ouranogyne*** S.Moore, *Andreasen 310* (UPS), —, —, AM266947[9], AM266859[9], —, AM267032[9], —. ***Pentanisia prunelloides*** Schinz., *Bremer et al. 4275* (UPS), —, —, AM266948[9], AM266860[9], —, AM267033[9], KT792995[4]. ***Rubia fruticosa*** Aiton. 1, *Rova T015* (GB), —, —, AF102475[18], —, —, —, —. ***Rubia tinctorum*** L. 1, *Jury 19809*, —, —, —, —, —, HE602444[12], —. ***Rubia tinctorum*** L. 2, *Bremer 3300* (UPS), —, —, FJ695421[15], —, —, —, —. ***Spermacoce capitata*** ex DC. 1, *Andersson 1908* (GB), —, —, EU543158[2], EU543069[2], —, AM939536[10], —. ***Spermacoce capitata*** ex DC. 2, *Quieroz et al 14261* (CTES), —, —, —, —, —, —, KF737032[20]. ***Spermacoce filifolia*** Perr. & Lepr. ex DC., *Dessein 881*, —, —, KT252882[8], KT252879[8], —, —, —. ***Spermacoce hispida*** L., *Wanntorp et al. 2667* (S), —, —, EU543162[6], EU543073[6], —, AM939540[10], AM933017[10]. ***Spermacoce ocymifolia*** Willd. 1, *Bremer 3340* (UPS), —, —, —, —, —, AM939462[10], AM932951[10]. ***Spermacoce ocymifolia*** Willd. 2, *Andersson et al. 2040* (GB), —, —, EU543108[6], —, —, —, —. ***Spermacoce prostrata*** R.D.Good, *Andersson et al. 2078* (GB), —, —, EU543163[6], —, —, AM939541[10], AM933012[10].

Appendix 2. List of morphological characters and characters states used in the morphological analyses for subtribe Anthosperminae.

1. Habit—perennial herb = 0; woody shrub/subshrub = 1. **2. Stem branching pattern—**erect (single stemmed) = 0; trailing (rooting at nodes)/prostate = 1. **3. Stem hairs—**hairy/shortly hairy = 0; glabrous = 1. **4. Leaf arrangement—** decussate = 0; in whorls of 3 = 1. **5. Hairs on leaves—** both surface hairy/shortly hairy = 0; upper surface hairy = 1; glabrous = 2. **6**. **Leaf size—** large and thin (30-80 X 12-25 mm) = 0; small and narrow (1.5-20 X 0.5-7 mm) = 1. **7. Petioles presence—** present/distinct petiole = 0; absent/obsolete petiole = 1. **8. Pedicel presence—** in terminal thyrsic/ thyrso-paniculate = 0; pedicellate = 1; sessile/subsessile = 2. **9. Number of Flower per node—** single = 0; paired = 1; in cluster of many = 2. **10. Corolla merosity—** 4-merous = 0; 5-merous = 1. **11. Carpophore presence—** absent = 0; present = 1. **12. Fruit dehiscence—** dehiscent = 0; indehiscent = 1. **13. Fruit calyx lobes presence—** absent = 0; present = 1. **14. Flowering season—** summer = 0; spring = 1; winter = 2; autumn = 3.