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A Phylogenetic Study of the Old World Asclepiadinae (Apocynaceae) Based on Chloroplast and Nuclear DNA Sequence Data

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The abstract and dissertation of David Chuba for the Doctor of Philosophy in Biology: were presented October 16, 2009, and accepted by the dissertation committee and the doctoral program.

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ABSTRACT

An abstract of the dissertation of David Chuba for the Doctor of Philosophy in Biology, presented on October 16, 2009

Title: A phylogenetic study of the Old World Asclepiadinae (Apocynaceae) based on chloroplast and Nuclear DNA sequence data

Relationships within the African *Asclepias* generic complex (Asclepiadinae, Apocynaceae) have for a long time been only a matter of intuitive speculation and generic delimitations have been diverse and rather contentious. Generic delimitation in this group has been based on morphological characters that are usually not exclusive to any particular clade or genus. The difficulty of identifying taxonomically useful morphological characters for inferring generic delimitations has led to differences in the emphasized characters by different taxonomists. This study is aimed at understanding phylogenetic relationships of species within the African *Asclepias* complex based upon the nuclear *PgiC* and three chloroplast DNA regions, *rpl16* intron, *trnC-rpoB* spacer and *trnS-G* spacer/*trnG* intron. The data were analyzed by maximum parsimony, maximum likelihood and Bayesian inference methods and hypothesis tests were performed to test the monophyly of the putative genera using the Shimodaira-Hasegawa test. Lastly maximum parsimony was used to reconstruct ancestral character states for species habitat preference and some morphological characters previously used for generic delimitations in the African *Asclepias* complex.

The African *Asclepias* complex is made up of two basal lineages, one containing only the Ethiopian highlands endemic species *Trachycalymma pseudofimbriatum* as the first diverging lineage and the other containing the rest of the African *Asclepias* sensu lato. Members of the *Gomphocarpus integer* group plus *G. tomentosus* and *G. filiformis* form the next diverging lineage sister to the remaining species. A number of genera are not monophyletic namely *Gomphocarpus*, *Pachycarpus*, *Stathmostelma*, *Xysmalobium*, *Trachycalymma*, *Schizoglossum* and *Aspidoglossum*. *Glossostelma* is monophyletic. Species still classified as *Asclepias* sensu lato show various, strongly supported affinities as follows: *A. aurea* is sister to *A. cucullata*, *A. randii* is sister to sample attributable to *Gen. indet. aff. Asclepias* (10917), *A. macropus* is sister to *A. praemorsa*, *A. densiflora* is sister to *Xysmalobium aceratoides* and *A. gibba* is sister to *A. disparilis*. The morphological characters optimized on the phylogenetic tree, were all homoplasious as indicated by multiple origins of character states. Ancestral characters states included stems branching, broad leaves, presence of nodal only inflorescences, pedunculate inflorescences and the *Gomphocarpus* type cucullate plus laterally flattened corona lobes.

A PHYLOGENETIC STUDY OF THE OLD WORLD ASCLEPIADINAE (APOCYNACEAE) BASED ON CHLOROPLAST AND NUCLEAR DNA SEQUENCE DATA

by

DAVID CHUBA

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The phylogeny of the African *Asclepias* generic complex (Apocynaceae) based on three chloroplast DNA regions

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ABSTRACT

Relationships among members of the African *Asclepias* generic complex (Asclepiadinae, Apocynaceae) have for a long time been only a matter of intuitive speculation and generic delimitations have been diverse and rather contentious. The difficulty of identifying taxonomically useful morphological characters for inferring phylogenetic relationship and generic delimitations has led to differences in the emphasized characters by different taxonomists. This lack of agreement on morphological characters has prompted a search for independent criteria to determine relationships of these plants. We infer phylogenetic relationships of the African

Asclepias complex based upon three non-coding chloroplast DNA regions, *rpl16* intron, *trnC-rpoB* spacer and *trnS-G* spacer/*trnG* intron. Sequence data were analyzed by maximum parsimony, maximum likelihood and Bayesian inference methods and hypothesis tests were performed to test the monophyly of the putative genera using the Shimodaira-Hasegawa test. We find that the African *Asclepias* complex is made up of two lineages, one containing only *Trachycalymma pseudofimbriatum* and the other containing all other African *Asclepias* sensu lato. These lineages form a polytomy with the American *Asclepias* sensu stricto clade. The American *Asclepias* sensu stricto outgroup clade is monophyletic. The large African clade has two sister lineages, one containing all sampled members of the *Gomphocarpus integer* group plus *G. tomentosus* and *G. filiformis* and another containing the rest of the African species. In the major African clade, most genera are not monophyletic namely *Gomphocarpus*, *Pachycarpus*, *Stathmostelma*, *Xysmalobium*, *Trachycalymma*, *Schizoglossum* and *Aspidoglossum*. *Glossostelma* is monophyletic. Species still classified as *Asclepias* sensu lato show various, strongly supported affinities as follows: *A. aurea* (Schltr.) Schltr. is sister to *A. cucullata* Schltr., *A. randii* S. Moore is sister to a sample attributable to *Gen. indet. aff. Asclepias* (10917), *A. macropus* (Schltr.) Schltr. is sister to *A. praemorsa* Schltr., *A. densiflora* is sister to *Xysmalobium aceratoides* (Schltr.) N.E. Br., and *A. gibba* Schltr. is sister to *A. disparilis* N.E. Br. The highly conservative SH test did not reject any apriori African *Asclepias* generic hypotheses considering the species sampled. The monophyly of the African *Asclepias* including *Trachycalymma pseudofimbriatum* was also not rejected by the SH test. The emergent lineages within the African *Asclepias* will be useful in testing many

hypotheses regarding evolutionary phenomena such as species pollinator interactions and defense traits and will also afford comparisons of such evolutionary trends with the related and well studied American *Asclepias* sensu stricto.

Keywords – Africa, *Asclepias*, Asclepiadinae, milkweed, *rpl16*, *trnC-rpoB*, *trnS-G/trnG*

INTRODUCTION

The African *Asclepias* complex consists of approximately 250 species in 18 of the 21 genera in the subtribe Asclepiadinae (Goyder 2001a), which belongs to the tribe Asclepiadeae within Apocynaceae subfamiliy Asclepiadoideae (Endress and Bruyns 2000; Liede 1997). These plants are restricted to the Old World, ranging from Sinai, and north to the Dead Sea, Arabian peninsula, covering most of sub Saharan Africa from Ethiopia and Sudan in north eastern part of Africa and Senegal and Gambia in north western part of African south to the Cape. However their diversity is centered in southern and eastern Africa (Liede 1997). They inhabit various habitats ranging from highland grasslands, open *Brachystegia*, mixed deciduous or scrub woodland and sometimes riparian woodland to disturbed sites, such as alongside roads.

Like many other members of the family Apocynaceae, these plants possess unusual and often brightly colored pentamerous, actinomorphic and bisexual flowers (Endress 1994; Fishbein 2001; Radford 1974). A notable feature of this group and all other Ascelpiadoideae flowers is that the petals, stamens, or their common base have appendages that are collectively termed "corona". Another special feature is a highly synorganised flower (Endress 1994) with parts that are physically and functionally united to form a structure known as a gynostegium, made up of the five stamens and the two carpels. An additional level of synorganization found in all Asclepiadoideae is the packaging within the anthers of the pollen into coherent masses called pollinia.

Potential synapormorphies for the subtribe Asclepiadinae include a corona made of strongly differentiated staminal and interstaminal parts and either an erect

(Liede 1997), decumbent, procumbent or ascending habit (Bruyns 1999). Relative to other Asclepiadoideae a non-twining habit is common to all members of the Asclepiadinae except *Pergularia* L., which has a twining habit that is probably symplesiomorphic. *Pergularia* has been placed in Asclepiadinae on the basis of chemical and molecular evidence (Fishbein 2001; Goyder et al. 2007; Liede-Schumann 1994; Liede-Schumann et al. 2005; Liede 1997; Rapini et al. 2003; Rapini et al. 2007).

Plants of the African Asclepiadinae have been poorly studied compared to the closely related American *Asclepias* s.s. A few studies that have involved the African *Asclepias* include for example investigation of pollination and ecology (Ollerton et al. 2003; Ollerton and Liede 1997; Shuttleworth and Johnson 2006; Shuttleworth and Johnson 2008) and also nectar systems due to their highly specialized and various nectar conducting systems (Kunze 1997). There have been far more numerous ecological, evolutionary, and developmental studies of species of the closely related *Asclepias* s.s., for example studies of the evolution of plant defense (Agrawal and Fishbein 2006; Agrawal et al. 2008; Agrawal et al. 2009), inflorescence design (Fishbein and Venable 1996; Willson and Price 1977) and reproductive ecology (Wyatt and Broyles 1994; Wyatt et al. 1992). Even though a few non phylogenetic studies have employed the African *Asclepias* complex, their results have not been analyzed in an evolutionary context so as to complement the various such studies conducted with the *Asclepias* s.s. Therefore understanding the systematics of African Asclepiadinae is crucial in complementing what is well known about the evolution of various phenomena such as defense, floral morphology, and vegetative morphology

and plant pollinator interactions in the *Asclepias* s.s. This study provides a basis on which such phenomena can begin to be analyzed for the African *Asclepias* complex.

Whereas the taxonomy of the better known American *Asclepias* sensu stricto has been fairly stable for the past half century, generic delimitations in the African Asclepiadinae have been diverse and rather contentious because of differential emphasis on variation in floral, vegetative, and fruit characters. For example Robert Brown (Brown 1810) separated *Gomphocarpus* from *Asclepias* based on absence of a tooth in the cavity of the corona lobe as well as the inflation and ornamentation of fruits in *Gomphocarpus*. However Nicholas Edward Brown (Brown 1904) took a different approach (Table 1) and argued that the presence or absence of a toothed corona lobe was not a defensible criterion for separating *Asclepias* from *Gomphocarpus*, as this would have placed closely related species in different genera (e.g. *A. tenuifolius* N.E. Brown without a tooth and *A filiformis* Benth. & Hook.f with a tooth) and required placement of some species in both genera (e.g. *A. coccinea* N.E. Br. in which the horn may or may not be present). Another example of difficulty generated by reliance on single floral characters is the treatment of *Stathmostelma* K. Schum. by Schumann (Schumann 1893), who circumscribed the genus to include species exhibiting broad translator arms (structures connecting two pollinia in adjacent anthers) on the pollinia (mass of attached pollen grains) (Table 1). According to Bullock this circumscription was problematic since the genus included species exhibiting a gradual series of variation from long and slender to short and broad caudicles. However, Goyder (Goyder 1998b) later retained all the species recognized by Schumann and circumscribed the genus based on more than one character, namely

pollinia with broadly winged, contorted translator arms and anther wings with convex margins and basal tails. Thus conflicting decisions of where generic limits should be placed have been based on different floral structures emphasized in classifications, a fact that has prompted and justifies a search for independent criteria to determine phylogenetic relationships of these plants [e.g. Goyder et al., (Goyder et al. 2007)].

Due to the mutual possession of similar overall floral morphology and an erect habit, species in the African *Asclepias* complex had for a long time been included under the otherwise American genus *Asclepias* (Brown 1810) (Baillon 1890) (Schlechter 1895 ; Schlechter 1896) (Schumann 1895) (Brown 1904; Brown 1909). A kind of turning point for the African members of the complex came in the 1950s and 1960s when Bullock carried out work for the *Flora of West Tropical Africa* and made an incomplete attempt at synthesis (Bullock 1952; Bullock 1953a; Bullock 1953b; Bullock 1954a; Bullock 1954b; Bullock 1955; Bullock 1963) (Table 1). He considered the genus *Asclepias* sensu stricto to be non-native to the African continent and to be exclusively New World in its natural distribution (*A. syriaca* L. of North America is the type species). Therefore, Bullock (Bullock 1952) proposed the exclusion of African species from *Asclepias* sensu stricto (from here on we refer to a circumscription of *Asclepias* limited to American species as "*Asclepias* sensu stricto" and a circumscription including all species of Asclepiadinae, except *Pergularia, Calotropis* R. Br., and *Kanahia* R. Br., as "*Asclepias* sensu lato") and their redistribution among other genera proposed earlier (Brown 1810; Brown 1811; Meyer 1838; Schlechter 1895 ; Schlechter 1896; Schumann 1893; Schumann 1895), as well

as genera that were to be newly proposed by him. In revising the generic taxonomy of African *Asclepias*, Bullock only considered groups in tropical Africa and never included extra-tropical species. Perhaps as a result, almost a decade later Dyer (Dyer 1975), in his "Genera of Southern African flowering plants", adopted N. E. Brown's (Brown 1909) much earlier classification for the approximately 60 southern African species belonging to the region by adopting an inclusive *Asclepias*, though he did not make his own systematic study of the group. Bullock's goal of redistributing the African species to other genera was never realized by him, but his approach to the taxonomy of the group has been extended by Goyder and Nicholas e.g. (Goyder 1998a; Goyder 1998b; Goyder 2001a; Nicholas and Goyder 1990; Nicholas and Goyder 1992).

Nicholas and Goyder (Nicholas and Goyder 1992) first contributed to the generic taxonomy of *Aslcepias* sensu lato by describing a new genus, *Aspidonepsis* Nicholas and Goyder, consisting of five species (Table 1). Goyder subsequently revised *Glossostelma* Schltr., with 12 species (Goyder 1995), *Stathmostelma*, with 13 species (Goyder 1998b), *Pachycarpus* E. Mey., with 15 species (of 37 total) in tropical Africa (Goyder 1998b), and *Trachycalymma* Bullock, with 10 species (Goyder 2001b). *Gomphocarpus*, with 20 species native to Africa, Arabia and adjacent territories south of the Dead Sea, was revised by Goyder and Nicholas (Goyder and Nicholas 2001). Other workers who have contributed important, modern revisions of African *Asclepias* sensu lato include Kupicha (Kupicha 1984), on *Aspidoglossum* E. Mey. and *Miraglossum* Kupicha, and Smith (Smith 1988), on southern African *Pachycarpus*.

Currently, there are 18 genera of the African *Asclepias* complex recognized by Goyder (Goyder 2001a). However, limits for some of the genera are certainly not firmly fixed. The most variable taxa, containing putatively distantly related species, are *Asclepias* and *Xysmalobium* R. Br., which are currently considered as "dustbins" for species yet to be assigned to segregate genera. African Species still named "*Asclepias*" will ultimately be transferred to other genera once their affinities are established in this or later studies. These are currently under study by Goyder and Nicholas (unpublished), and will result in the reassignment of all African species remaining in *Asclepias*. Some *Xysmalobium* species that form a monophyletic group will remain in that genus, although others are presumed to belong elsewhere (Goyder 2001a). Species residing in these genera could potentially be placed in at least seven others (Goyder 2001a). Phylogenetic study will therefore be useful in providing more insight into the placement of these "orphan" species and speed up work towards a global taxonomic synthesis of the Asclepiadinae.

In addition to the recent attempts at delimiting genera based on multiple morphological characters, molecular approaches to resolving the African *Asclepias* phylogeny may aid in identifying morphological synapomorphies that have thus far remained elusive. So far only very few studies employing molecular phylogenetic analyses have included species of the African *Asclepias* complex, i.e. Potgieter and Albert (Potgieter and Albert 2001) – who focused on the family level; Rapini et al. (Rapini et al. 2003) – who focused on subfamily Asclepiadoideae; Fishbein, et al. (submitted) – who focused on the American *Asclepias,* and Goyder et al. (Goyder et al. 2007) – the first study entirely focused on the African Asclepiadinae. Except for

Fishbein et al (submitted) who used *rpl16* intron, *trnC-rpoB* spacer and *trnS-G* spacer/*trnG* intron regions, these other studies mostly used the same chloroplast DNA (cpDNA) sequences to infer phylogeny. Potgieter and Albert (Potgieter and Albert 2001) used two non-coding cpDNA regions (*trnL* intron and *trnL-F* intergenic spacer) and included 152 species of Apocynaceae representing all major tribes of the family. However, they included only species of two genera from the African *Asclepias* complex. Their analysis resolved a strongly supported monophyletic Asclepiadinae with *Calotropis procera* (Aiton) W.T. Aiton as sister to the rest of the African (3 spp.) and North American (3 spp.) taxa included. Rapini et al. (Rapini et al. 2003) used the same two cpDNA regions and included 111 species of Asclepiadoideae representing the major lineages of the subfamily. Nine genera (one species each) of the African *Asclepias* complex were represented. Their results, though weakly supported, suggested that Asclepiadinae was monophyletic with *Pergularia* as sister to the rest of the taxa. Goyder et al. (2007) also used the *trnL* intron and *trnL-F* spacer, and added the adjacent *trnT-L* spacer and the nuclear ribosomal internal transcribed spacer (ITS) region. They sampled 65 species in 21 genera of Asclepiadinae, of which 60 belonged to the African *Asclepias* complex. The sampling included the following genera: *Calotropis, Cordylogyne* E. Mey., *Fanninia* Harv., *Glossostelma, Kanahia, Lagarinthus* E. Mey., *Margaretta* Oliv., *Miraglossum*, *Pergularia*, *Stenostelma* Schltr., *Woodia* Schltr., and one undescribed genus (1 sp. each); *Aspidonepsis* (2 spp.), *Stathmostelma* and *Trachycalymma* (3 spp. each), *Aspidoglossum* and *Schizoglossum* E. Mey. (4 spp. each), *Gomphocarpus* (6 spp.), *Xysmalobium* (8 spp.), *Pachycarpus* (10 spp.), and African *Asclepias* sensu lato (8 spp.). This study found some surprising

results, including the non-monophyly of a number of African genera of Asclepiadinae, though some with only weak statistical support. Fishbein et al. (submitted) sampled 19 species of the African *Asclepias* complex representing 12 segregate genera. However, the sampling was not enough to test the monophyly of these genera.

In this study, we conduct more densely sampled molecular phylogenetic analyses of African *Asclepias* sensu lato in order to improve the delimitation of genera, including evaluating the need to erect new genera in Asclepiadinae. This approach complements and provides a basis for evaluating approaches employing morphology for generic delimitation and also expands on earlier molecular studies that had limited sampling. Our study had two specific aims: firstly, to employ the chloroplast *rpl16* intron, *trnC-rpoB* spacer, and *trnS-G* spacer/*trnG* intron regions (Shaw et al. 2005b; Shaw et al. 2007; Small et al. 1998) to determine phylogenetic relationships of the African *Asclepias* complex and secondly, to evaluate the currently proposed generic concepts. We thus tested the hypotheses: 1) that the African species of *Asclepias* sensu lato are monophyletic and that there is a clear African - American split within the Asclepiadinae, as has been suggested by previous studies such as Potgieter and Albert (Potgieter and Albert 2001), Rapini et al. (Rapini et al. 2003) and Goyder et al. (Goyder et al. 2007); and 2) that the putative genera accommodating the species in the African *Asclepias* complex are monophyletic.

MATERIALS AND METHODS

Taxon Sampling

A total of 86 species were sampled from 13 genera within the African *Asclepias* generic complex and five outgroup genera (See Appendix A). These were selected to represent (a) recognized genera and unassigned species (b) character variation previously utilized to delimit taxa and (c) different geographic regions around Africa. The DNA samples were obtained from Royal Botanic Gardens-Kew, United Kingdom (57 samples), South African National Botanical Institute-Cape Town, R.S.A. (18 samples), field collections (two samples) and 12 samples that were already available (Fishbein et al. submitted).

Outgroups included nine species of American *Asclepias* s.s. (see appendix A) and one species each of *Kanahia*, *Pergularia* and *Calotropis*, which have been shown to be the earliest diverging Asclepiadinae (Goyder 2001a; Rapini et al. 2003), Fishbein et al., submitted). To root the phylogeny of Asclepiadinae, a more distant outgroup *Cynanchum ligulatum* (Benth.) Woodson from another subtribe, Cynanchinae K. Schum, was included. DNA sequences were already available for 39 of the species sampled (Appendix A).

DNA extraction amplification and Sequencing

DNA was extracted using either the Qiagen DNeasy Plant Mini kit (PE Biosystems), or a modification of the small-scale CTAB extraction method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) was utilized to obtain sequencing templates for three cpDNA regions (*rpl16* intron, *trnC-rpoB* spacer, and *trnS-trnG*

spacer/*trnG* intron) (Shaw et al. 2005b; Shaw et al. 2007; Small et al. 1998). These regions were chosen based on previous analyses of the phylogenetic utility of the most often used non-coding cpDNA regions for systematic investigations (Shaw et al. 2005a; Shaw et al. 2007). In these studies, the *trnS*^{GCU}-*trnG*^{UUC} and the *rpoB-trnC*^{GCA} intergenic spacers were among the five spacers that provided the most potentially informative characters (PICs). Further the *trnS-trnG* spacer combined with the *trnG* intron (*trnS-trnG-trnG*) was found to provide the greatest number of PICs compared to all other regions. The *rpl16* intron is a relatively more slowly evolving region and this was hoped to provide better basal resolution. The thermal profile for PCR amplification of *trnS-G* region from genomic DNA was as follows: initial denaturation at 95° C for 2 min, followed by 30 cycles of 95° C for 1 min, 52.9° C for 1 min, 65° C for 4 min, and ending with a final extension at 65° C for 8 min. The *rpl16* and *trnC-rpoB* regions were also amplified with the same general profile except for the annealing temperatures, which were varied as follows: 50° C (1 min) for *rpl16* and 51° C (1 min) for the *trnC-rpoB* region. Amplification conditions generally consisted of 50 µl reactions containing approximately 1µL (~10 - 20ng) of DNA, primer pair with 1 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and a combination of 1× PCR reaction buffer plus 0.25-0.5 U *Taq* DNA polymerase both either obtained from Promega (Madison, Wisconsin, USA) or Fermentas (Hanover, MD, USA).

The templates were either sequenced using the ABI BigDye® Terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc. California) and analyzed using an ABI 3700 Genetic analyzer sequencer (Oregon Health Sciences University Vollum Lab) or using the Beckman CEQ kit (Beckman Coulter, Fullerton,

CA) and analyzed using a Beckman CEQ 8000 DNA sequencer (Mississippi State University Life Sciences Biotechnology Institute). Universal and specially designed internal primers (Table 2) were utilized to optimize double stranded sequencing. The general cycle sequencing profile was as follows: initial denaturation at 80° C for 4 min, followed by 25cycles of 96° C for 10 sec, 50° C for 5 sec. and 60° C for 4 min.

Data Analysis

DNA sequences were assembled using SeqMan™ II (DNASTAR 1999), and manually aligned using the sequence alignment editor, Se-Al v2.0 (Rambaut 2007) in combination with MacClade (Maddison and Maddison 1992). For model-based analyses, the appropriate nucleotide substitution models for each region and the combined dataset were determined using ModelTest 3.7 (Posada and Crandall 1998) and MrModeltest 2.3 (Nylander 2004). ModelTest 3.7 utilizes both a hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) for model selection. MrModeltest is a modification of Modeltest 3.6 and is used to compare 24 models (the only ones implemented in MrBayes) instead of the 56 models tested by Modeltest. The Partition Homogeneity test (Farris J.S. 1994) was employed using PAUP*v4.0b10 (Swofford 2001) to examine congruence in the phylogenetic signal of the three regions.

Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP*v.4.0b10 (Swofford 2001) and Bayesian phylogenetic inference with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). For parsimony analysis, alignment gaps were examined for potentially

informative insertion or deletion events (indels). Phylogenetic analyses were performed utilizing two coding schemes, one in which gaps were treated as missing data and another in which indels were recoded as multistate characters with the sequence data in the indel regions excluded from the alignment. A total of 12 indels was coded. Parsimony analysis of the combined dataset was conducted using a heuristic search with tree bisection and reconnection (TBR) branch swapping, 10,000 random addition sequence replicates, 10 trees held at each step and no more than 100 trees saved in each replicate. To further search for most parsimonious trees (MPTs), a parsimony ratchet analysis (Nixon 1999) was conducted using the PAUPRat program (Sikes and Lewis 2001) in combination with PAUP*. The default setting of 25% characters re-weighted per iteration was utilized. A total of 201 trees were saved for each of the 20 ratchet replicates. These were filtered to retain only the best trees, and then summarized with a strict consensus tree. Support for each node of the inferred phylogeny was estimated by nonparametric bootstrap analysis (Felsenstein 1985) (5000 bootstrap replicates) using a heuristic search with TBR branch swapping, 10 random addition sequences per bootstrap replicate, 1 tree held at each step during each random addition sequence and 10 trees saved per replicate.

Maximum likelihood (ML) analysis was conducted using the maximum likelihood ratchet (Morrison 2007) as implemented in PRAP2 (Muller 2007) under the GTR+I+G nucleotide substitution model suggested by the hierarchical likelihood ratio tests (hLRT) and Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Crandall 1998). PRAP2 generates command files that are executed by PAUP. The search procedure was as follows: (i) a starting tree was obtained by calculating a

BioNJ tree using Logdet distances, followed by one round of Nearest Neighbor Interchange (NNI) search (Moore et al. 1973), followed by optimization of substitution model parameters and one round of (subtree pruning-regrafting) SPR branch swapping; (ii) alternating branch swapping on the original matrix and on a matrix with 25% of characters upweighted, saving only one tree; and (iii) SPR branch swapping on the single tree saved to obtain the ML Tree. Ten ratchet iterations were performed. This method has been found to be the most successful for datasets with low levels of phylogenetic signal (Morrison 2007), as is the case for our dataset.

Bayesian analysis was conducted with MrBayes version 3.2.1 (Huelsenbeck and Ronquist, 2001). The combined 3-gene dataset was partitioned by gene region and the best nucleotide substitution model, GTR + G, applied to each of the three data partitions, as suggested by the hLRT and AIC tests in MrModeltest 3.06. Seven chains (one cold and six heated) were used and run for a total of 11,000,000 generations, under the program's default priors on model parameters and branch lengths. Trees were sampled from the cold chain every 1000 generations. Two Markov chain Bayesian analyses were performed simultaneously, each starting with a different randomly selected initial topology. To assess convergence of the chains, the standard deviation of split frequencies were checked periodically until a frequency of less than 0.01 was achieved, indicating convergence. Stationarity and convergence of model parameters were also assessed using Tracer version1.4 (Rambaut and Drummond 2007). The log likelihood plot of the sampled trees was examined and trees in the 'burn-in' phase discarded in each run so that approximately only trees in the stationary phase of the chain were considered. A majority-rule consensus of the

remaining trees was then computed. Clade support for the inferred phylogeny was estimated by Bayesian posterior probabilities.

Hypothesis Tests

The nonparametric Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa 1999) as implemented in PAUP* (Swofford 2001) was utilized to test the monophyly of African *Asclepias* sensu lato and of putative genera of the complex. The test was implemented for each of the hypotheses of generic monophyly by inputting the maximum likelihood tree and the specific constrained tree for each hypothesis. Each alternative hypothesis tree was constructed by initially establishing the appropriate constraint using the graphic display in MacClade (Maddison and Maddison 1992) and then swapping using SPR branch swapping algorithm under the GTR+I+G nucleotide substitution model in PAUP* to find the ML tree under the constraint. These hypotheses were evaluated at $\alpha = 0.05$ against a null distribution based on 10000 replicates analyzed with the RELL method.

RESULTS

The combined aligned sequences of *trnS-trnG-trnG, rpl16* and *trnC-rpoB* sequences resulted in 4362 characters (Table 3) for 86 taxa and of these about 1.2% of the cells had missing information, whereas 247 positions were ambiguously aligned and were therefore excluded from all the analyses. The null hypothesis of homogeneity of the phylogenetic signal among the three DNA regions was not rejected as indicated by the partition homogeneity test (*trnS-trnG-trnG* vs *trnC-rpoB*,

$p = 0.373$; *trnC-rpoB* vs *rpl16*, $p= 0.352$; *trnSG* vs *rpl16*, $p=0.847$). Therefore sequence data from the three plastid gene regions were combined for the phylogenetic analyses.

For the MP analysis the dataset without coded indels contained 4238 characters of which 176 (4.4%) were parsimony informative. Coding indels as multistate characters and excluding the indel positions from the data matrix resulted in 3996 characters, 188 (4.7%) of which were parsimony informative. The MP analysis of the three-region dataset yielded 99447 shortest trees L= 723 steps, CI= 0.8285, RI= 0.7825). The parsimony ratchet analysis also yielded shortest trees of the same length (723 steps). The strict consensus of all MPTs recovered in the parsimony analysis is shown in Fig. 1.

The ML tree recovered from the maximum likelihood ratchet analysis had a ln likelihood of –10723.95794 and is shown in Fig. 2. In the Bayesian analysis, all model parameters appeared to attain stationarity as indicated by examination of the model likelihood trajectory in Tracer, which also suggested a burn in phase of 1,100,000 generations, therefore the first 11,000 samples of trees and parameters were discarded. All effective sample sizes of parameters were sufficiently high (above 100) to conclude adequate sampling from the posterior probability space. The potential scale reduction factor values for all parameters were 1.000 or very close to this value, indicating convergence of the two runs. Thus, we combined the post burn-in trees into a single pool of 1,980,000, which we used to calculate the posterior probabilities for clades which are indicated on the ML tree (Fig. 2).

The overall structure of the topology recovered by the ML analysis was similar to the MP tree in most of the terminal relationships, but had additional basal resolution not present in the MP tree, as explained below.

The African *Asclepias* generic complex is resolved within a strongly supported (BS 100, PP 100) clade (O) that also includes American *Asclepias* sensu stricto (Figs. 1 & 2). This clade is sister to the *Kanahia-Calotropis* clade with which it forms a well supported (BS 93, PP 100) more inclusive clade (X). The American *Asclepias* s.s. outgroup clade is resolved as monophyletic in ML (PP 99) but in the MP analysis (Fig 1) there were two clades of the American *Asclepias* whose relationship to each other was unresolved though each was well supported (Clade NS: BS 100, PP 100 Clade NN BS 80, PP 98). The African *Asclepias* complex is not monophyletic, being made up of two lineages--one containing only *Trachycalymma pseudofimbriatum* Goyder and the other containing all other African *Asclepias* sensu lato (clade M; Fig. 1 and Fig. 2; BS 56, PP 97), which together form a polytomy with the American *Asclepias* sensu stricto clade in the ML tree (Fig. 2) or with two unresolved clades of the American *Asclepias* sensu stricto in the MP tree (Fig.1). The large African clade has two sister lineages, one containing the *Gomphocarpus integer* (N.E. Br.) Bullock group (Goyder and Nicholas 2001) plus *G. tomentosus* and *G. filiformis* (Clade A; Fig. 1 and Fig. 2; BS 75, PP 100) and another containing the rest of the African species (clade L, Fig. 1 and Fig. 2; PP 95). Within clade L there are a number of smaller clades that form a polytomy. However, the ML analysis yielded a number of more inclusive clades; J1, K1, X1, Y1 and W1 (Fig 2) that are not found either in the MP

analyses. Support for these larger clades by maximum likelihood bootstrap was not estimated due to computational constraints.

A notable result of the analyses is that in the major African clade (i.e., clade M), most genera are not monophyletic. There is a clade of seven *Gomphocarpus* accessions (clade H) that is strongly supported, but with no affinity to the *Gomphocarpus integer* group plus *G. tomentosus* and *G. filiformis* clade (A) or any other sampled species of *Gomphocarpus*. A third *Gomphocarpus* clade is well supported (clade T; BS 85, PP 100), whereas the remaining two species of *Gomphocarpus* are not resolved with any other *Gomphocarpus* clade. The three sampled species of *Glossostelma* (clade U) are strongly supported (BS 99, PP 100) as monophyletic. Four *Stathmostelma* species form a moderately well supported (BS 60, PP 94) clade (B), whereas two subspecies of *S. spectabile* (N.E. Br.) Schltr. that are strongly supported (BS 91, PP 100) as sister in clade C are unresolved with respect to clade B. In the ML tree *Stathmostelma verdickii* De Wild. is weakly resolved as sister to *Margaretta rosea* Oliv., a relationship that is absent in the MP tree. Three *Pachycarpus* species (clade E) are placed in one clade with moderate support (BS 61, PP 100), whereas two others, *P. bissaculatus* (Oliv.) Goyder and *P. lineolatus* (Decne.) Bullock are placed in a second clade (BS 93, PP 100), and the placement of *P. campanulatus* (Harv.) N.E. Br. var. *sutherlandii* N.E. Br. is unresolved. Two of the sampled *Trachycalymma* species form a well supported (BS 91, PP 100) clade (K) and have weak parsimony bootstrap support, but strong Bayesian support (BS 62, PP 99) as sister (clade S) to the strongly supported *Pachycarpus bissaculatus-P. lineolatus* clade. One *Trachycalymma* species, as indicated above, is resolved outside the main

African clade (M). Of the *Xysmalobium* species sampled, four are resolved within one well supported (BS 95, PP 92) clade (clade F) whereas two others are placed in a clade (R) with *Asclepias densiflora* N.E. Br., though lacking bootstrap support (PP 88). *Schizoglossum hamatum* E. Mey. and *S. hilliardiae* Kupicha are strongly supported (BS 86, PP 100) as sister and are nested in a *Schizoglossum-Aspidoglossum* clade containing six *Aspidoglossum* species. This clade has weak bootstrap support but strong Bayesian support (clade I; BS 53, PP 97). Interestingly, "*Schizoglossum alpestre*" K. Schum. (which has not been included in *Schizoglossum* but not assigned to any other genus (Kupicha 1984) and a sample attributable to a putative new genus (*Goyder 4895*), are well supported (clade D; BS 89, PP 99) as sister in yet another lineage within clade L. One species of *Aspidoglossum* (*A. heterophyllum* E. Mey.) is resolved with weak bootstrap support (BS 67, PP 96), but moderate Bayesian support, as sister to the only sampled species of *Aspidonepsis* [*A. flava* (N.E. Br.) Nicholas & Goyder]. Species still classified as *Asclepias* sensu lato showed various, strongly supported affinities as follows: *A. aurea* (Schltr.) Schltr. is sister to *A. cucullata* Schltr., *A. randii* S. Moore is sister to sample attributable to a second putative new genus Gen. indet. aff. *Asclepias* (10917), *A. macropus* (Schltr.) Schltr. is sister to *A. praemorsa* Schltr., *A. densiflora* is sister to *Xysmalobium aceratoides* (Schltr.) N.E. Br., and *A. gibba* Schltr. is moderately supported as sister to *A. disparilis* N.E. Br.

The SH test did not reject any a priori African *Asclepias* generic hypotheses ($P > 0.05$; Table 4) considering the species sampled. The monophyly of the African *Asclepias* including *Trachycalymma pseudofimbriatum* was also not rejected by the SH test.

DISCUSSION

Maximum parsimony and maximum likelihood analyses produced similar topologies (Figs 1, 2) with high support for some clades. The overall topology of the estimated phylogeny was mostly consistent with the topology found with much more limited sampling by Goyder et al. (Goyder et al. 2007). The species sampling in the two studies was nevertheless different with only 32 species included in that study, also sampled in our study. The basal relationships are consistent with the findings of Potgieter and Albert (Potgieter and Albert 2001) whose results strongly supported Asclepiadinae as monophyletic with *Calotropis procera* as sister to the rest of the African (3 spp.) and North American (3 spp.) taxa included. Our basal resolution also supports Rapini et al. (Rapini et al. 2003)'s results (with 12 Asclepiadinae included) which suggested, though weakly, that Asclepiadinae was monophyletic with *Pergularia* as sister to the rest of the Asclepiadinae they sampled.

A surprising result is that the African members of *Asclepias* sensu lato are not resolved as unequivocally monophyletic owing to the unresolved position of *T. pseudofimbriatum* (Figs. 1 and 2). However, the SH test produced a non significant ($p= 0.7764$, Table 4) result for the difference between this topology and the constrained tree in which the African *Asclepias* including *T. pseudofimbriatum* was monophyletic, leaving open the possibility that the African clade may still being monophyletic even though the clade including all the species of the African *Asclepias* complex but excluding *T. pseudofimbriatum* is weakly supported by MP bootstrap (BS 56) but strongly supported by Bayesian support (PP 97). This resolution differs from Fishbein et al (accepted) in which *T. pseudofimbriatum* was resolved as sister to a

clade consisting of two well supported monophyletic sister lineages of the American *Asclepias* sensu stricto and the African *Asclepias* complex respectively. The outcome may have been due to a narrower sampling of the African *Asclepias* species in that study. *Trachycalymma pseudofimbriatum* was not sampled by Goyder et al (Goyder et al. 2007), the only other study focused on old world Asclepiadinae phylogeny. *Trachycalymma pseudofimbriatum* is endemic to the highlands of southern Ethiopia and is restricted only to montane grasslands at about 2700 m (Goyder 2001b) whereas the other sampled representatives of the genus, *T. buchwaldii* (Schltr. & K. Schum.) Goyder, *T. pulchellum* (Decne.) Bullock and *T. foliosum* (K. Schum.) Goyder, are all found in lower elevations (900–2400 m) in grasslands or open woodlands of west to southern tropical Africa. Since these two species are embedded with species of other genera in clade P (Fig 1) to the exclusion of *T. pseudofimbriatum*, the similarity of these lineages in vegetative and floral morphology is surprising. Complete interpretation of this phenomenon is not possible until the exact relationship of the three lineages in the polytomy (Clade O, Figs. 1 and 2) and the basal lineages within the rest of the African *Asclepias* (clade M, Figs. 1 and 2) become clear. *T. puchellum*, and *T. foliosum* were treated as conspecific by Bullock, whereas Goyder (2001), considered their apparent similarity as only due to convergence in some vegetative characters such as non-succulent linear to lanceolate leaves. In this study, *T. foliosum* and *T. puchellum* are well supported as sister indicating that their similarity in vegetative characters is due to common ancestry. Whereas *T. pulchellum* is restricted to central parts of Africa between 10° S and 15°S latitudes and west of 30°E longitude, *T. foliosum* is sympatric with it but also extends

further east of the 30° longitude and north to West Africa as far as the 10°N latitude (Goyder 2001b). Ecologically they both occur mostly in open, mixed deciduous woodland or occasionally in grassland. In contrast, *T. buchwaldii* which is weakly resolved as sister to *Stathmostelma pauciflorum* is found in grassland or *Brachystegia* woodland but usually on steep rocky hillsides.

In Goyder et al. (Goyder et al. 2007), two species of *Trachycalymma* were included in the results presented. The placement of *T. foliosum* as sister to *P. lineolatus* in Goyder et al. (Goyder et al. 2007) is supported by this study. Whereas *T. buchwaldii* was weakly resolved as sister to *Stathmostelma verdickii* in Goyder et al (Goyder et al. 2007), it is also only differently resolved with weak support as sister to *Stathmostelma pauciflorum* in our study. It will be necessary to further test the placement of *Trachycalymma* by also sampling other species especially *T. fimbriatum* which though endemic to Zimbabwe and Mozambique, is morphologically very similar to *T. pseudofimbriatum*, which is endemic to Ethiopia (Goyder and Nicholas 2001).

The phylogenetic relationships of *Gomphocarpus* suggest that the taxonomy of the genus needs to be revisited at least in relation to one clade (A). All sampled species of the informal *Gomphocarpus integer* group (Goyder 2001a) plus *G. tomentosus* and *G. filiformis* lineage (clade A, Figs. 1 & 2) are strongly supported especially by Bayesian PP as a sister clade to the rest of the African *Asclepias* complex. Ecologically within the clade A, *G. Stenophullus* and *G. integer* occur in similar semi arid open rocky ground and disturbed areas from Tanzania north to southern Ethiopia whereas *G. tenuifolius* is restricted to granite kopjes further south in

Zimbabwe and may have evolved in this type of habitat. The closely related *G. tomentosus* subsp. *tomentosus* is also geographically restricted to the southern part of Africa though it is ecologically distinct growing mostly on sand in open or disturbed areas. However, the unsampled subspecies (*G. tomentosus* subsp. *frederici*) is ecologically comparable to *G. tenuifolius* being apparently restricted to granite outcrops in Angola (Goyder and Nicholas 2001). *Gomphocarpus filiformis* which is sister to the rest in this group occurs in extremely arid environments in parts of the Namib Desert and the Karoo (Goyder and Nicholas 2001). This evolutionary scenario would suggest that clade A may have either been widespread or originated in southern Africa and spread north. These hypotheses remain to be tested by biogeographic analysis. However the resolution of clade A (Fig1) as sister to clade L may turn out to be equivocal with more data since there is no MP bootstrap support for clade L. Only one species resolved in clade A (*G. tomentosus*) was sampled by Goyder et al., (Goyder et al. 2007). However, it was resolved in that study as sister to a clade of *G. abbyssinicus, G. fruticosus and G. physocarpus* group but without strong support.

In another well supported (BS 87, PP 100) *Gomphocarpus* clade (clade H, Figs. 1 & 2), there are two subclades. One clade (HO) contains some members of the *G. physocarpus* E. Mey. group (Goyder and Nicholas 2001) plus one member of the *G. phillipsiae* (N.E. Br.) Goyder group (*G. abbyssinicus*). The other clade (HI) contains putative subspecies of *G. fruticosus* (L.) W.T. Aiton, but surprisingly also *G. physocarpus*, which is even more closely, related to *G. fruticosus* subp. *fruticosus* and subsp. *rostratus* than is the putative conspecific taxon *G. fruticosus* subsp. *decipiens* (N.E. Br.) Goyder & Nicholas. *G. fruticosus* subsp *decipiens* could therefore either be

elevated to species status or *G. physocarpus* be sunk under *G. fruticosus* and a morphological analysis employed to identify morphological synapomorphies for theses species. There is no clear ecological distinction between species in clade HI and those in clade HO. However, the two clades are geographically distinct. All species in clade HO have a more northern distribution from Northern Zambia and central Angola to as far north as northward as Eritrea in N E Africa and Guinea in West Africa. Species in the HI clade have a southern distribution from Western Angola, Zambia, Southern Malawi and Mozambique, south to the Cape Province of South Africa.

The strongly supported clade T (BS 85, PP 100) coincides with the *G. glaucophyllus* Schltr. group (Goyder and Nicholas 2001), characterized by unbranched annual stems and thickened non-tuberous, but woody rootstocks (Goyder and Nicholas 2001). This clade occurs in the montane grasslands and the *Brachystegia* belt that spans south central Africa with *G. glucophyllus* being the most widely distributed in terms of north to south spread, extending from South Africa north to Uganda in fire prone habitats (Goyder and Nicholas 2001). The other species only extend south from Zimbabwe to Tanzania in the north. The current resolution of *Gomphocarpus* lends support to the earlier suggestion of Fishbein's then unpublished results (1996), quoted in Goyder and Nicholas (Goyder and Nicholas 2001), that *Gomphocarpus* was key to understanding the evolution of the old world and new world parts of the *Asclepias* complex. Even though the basal relationships of the *Gomphocarpus* subclades is not resolved, the SH test indicates that constraining the clades to monophyly produces a

non-significantly different topology (p= 0.2450, but see discussion about the SH test below).

Whereas four *Stathmostelma* species are well supported (BS 60, PP 94) in one core clade (B), the resolution of *Stathmostelma spectabile* subsp. *frommii* (Schltr.) Goyder in Figs 1 and 2). and *S. spectabile* subsp. *spectabile* (*Stathmostelma spectabile* in Figs 1and 2) in one well supported (BS 91, PP 100) clade (C), though with an ambiguous relationship to the former group, is consistent with their close relationship (Goyder 1998b). The non resolution of these *S. spectabile* subspecies in relation to the other congeners may be reflected in their unique characters within the genus, such as their more broadly ovate leaves (more than 4 cm wide) whereas the other species have linear to lanceolate leaves (Goyder 1998b). Ecologically all the *Stathmostelma* species sampled are typically found in seasonally waterlogged grasslands in drier parts of eastern and central tropical Africa with however *S. spectabile* subspecies being frequently associated with limestone (Goyder 1998b).

The resolution of *Margareta rosea* as sister to *Stathmostelma verdickii* in the ML tree, though weakly supported, is interesting. However, this relationship is absent from the MP tree and owing to the weak support, confident interpretation will require more data. Furthermore the SH-test did not reject the monophyly of *Stathmostelma* species including *S verdickii* but excluding *M. rosea* (p= 0.5419, Table 4). *S. verdickii* was excluded by Goyder from *Stathmostelma* (Goyder 1998b) and he suggested that it was allied to *Gomphocarpus longissimus* but did not formally place there. The relationship of *S verdickii* to *Gomphocarpus* is, however, not resolved here.

Though three *Pachycarpus* species (Fig. 1, clade G) are placed in one clade, their relationship to other species is unresolved, which might suggest a possibility of the fourth one *P. campanulatus* var. *sutherlandii* being supported as part of this group (Fig. 1clade E) with additional data. This possibility is also suggested by the SH-test which failed to reject the hypothesis of a *Pachycarpus* clade including all sampled species (p= 0.3434, Table 4). It is worth noting that all the *Pachycarpus* species occur in upland grasslands but Clade G (Fig. 1) species are broad leaved and restricted to highland areas over 1000 m in East Africa and northern parts of Central Africa, whereas *P. campanulatus* is a narrow leaved, southern African species occurring in a broader altitudinal range from as low as 500m to over 1000m. The remaining species, *P. bissaculatus* and *P. lineolatus* (Fig.1, within clade S), are here shown to be more allied to species of other genera than to other congeneric species. One morphological characteristic in support of this resolution is the nature of the rootstocks in the two species, which consist of a bunch of fleshy, fusiform, horizontal roots that lie shallowly, just below the soil surface, as opposed to the other species, where rootstocks are slender, deep growing, vertical tubers (Goyder 1998a). *P. bissaculatus* and *P. lineolatus* are also the most widespread species of *Pachycarpus* sensu Goyder (Goyder 1998a), with the northern limit of *P. lineolatus* in Cote d' Ivoire in the west and Sudan in the east and ranging south as far as Angola on the west and Malawi on the east and the northern limit of *P. bissaculatus* in Guinea Bissau in the west and southern Sudan and Ethiopia in the east ranging southward as far as Angola in the west and Mozambique in the east.

Of the *Xysmalobium* species sampled, the four within the well supported (BS 95, PP 92) clade F should definitely constitute the group that retains the name *Xysmalobium* since it contains the type species *X. undulatum* (L.) W.T. Aiton. Except for *X. undulatum* which is only known from South Africa (Langley 1980), species in this clade have a mostly tropical distribution. The SH-test did not reject the hypothesis that *Xysmalobium* species sampled formed a monophyletic group ($p = 0.6090$, Table 4) leaving open the possibility that, with additional data, a number of taxa in this group might turn out to be part of this core group.

The genus *Aspidoglossum* may be paraphyletic since two of the *Schizoglossum* species are nested within the Aspidoglossum clade (I). The resolution of these two species as sister to *A. breve* within clade 'I' (BS 53, PP 97) suggests the possibility of more *Schizoglossum* species that are not sampled species being closely related to some *Aspidoglossum* species. This has important consequences for the circumscription of the two genera and new generic delimitations of species may have to be considered. Even though the resolution within clade 'I' is poor and it is not yet entirely clear how the individual species are related, the resolution of *Aspidoglossum heterophyllum* as sister to the sole sampled species of *Aspidonepsis (A. flava)* confirms at least the close relationship of one species of *Aspidonepsis* to *Aspidoglossum* as earlier suggested by Nicholas and Goyder (Nicholas and Goyder 1992). For "*Schizoglossum alpestre*", strong support as sister to the putative new genus represented by Gen. indet. aff. *Asclepias* (10488), in yet another unresolved lineage within clade L, seems to corroborate the proposed exclusion from *Schizoglossum* (Kupicha 1984). Kupicha, however, did not place the species in another genus. The non significant result (Table

4) for the test of monophyly for both *Schizoglossum* and *Aspidoglossum* leaves open the possibility of exclusive monophyly for both genera.

A few affinities for a number of species still classified as *Asclepias* sensu lato have also emerged, without however their relationships to other taxa being resolved. *A. aurea*, *A. cucullatum* and *A. randii* were considered as closely allied, based on corona lobes that are attached to the staminal column only near the base and the rest of the proximal portions continuing as well developed free lobes placed between the anther wings (Goyder 2001b). All three species also occur in either montane (*A. cucullata* and *A. randii*) or highveild (*A. aurea*) grasslands (Burrows and Willis 2005; Nicholas 1982). The close relationship of *A. aurea* and *A. cucullatum* is well supported (BS 80/PP 99) here. However, whether *A. randii* is a closely related to these two species is unclear as it is only very strongly supported (BS 91/PP 100) as sister to the putative new genus represented by Gen. indet. aff. *Asclepias* (10917).

The phylogenetic relationships between the different recovered clades within the main African Asclepiadinae clade (Clade L Figs. 1 and 2) still remain unclear due to lack of basal resolution within the clade by the chloroplast regions utilized in our study. Even though our analyses retrieved some moderately to strongly supported subclades of the African Asclepiadinae, the interrelationships of many of these subclades were not resolved. Furthermore most internal nodes in the tree are poorly supported, whereas the basal branches outside the African *Asclepias* clade 'L' were mostly strongly supported. Nuclear DNA sequences might improve resolution and reveal relationships not currently resolved. This assertion may be supported by the fact that analyses of separate data sets (not presented) did not independently recover

most relationships that appear in the combined analysis of all three loci. Further, some weakly supported relationships in a two-gene analysis (not shown) achieved stronger support in the three gene analysis.

A number of possible reasons are advanced to explain poor basal resolution as seen in this study, including lack of adequate information (Walsh et al. 1999), inappropriate phylogenetic methods (models of evolution assumed) or conflicting phylogenetic histories of the different combined DNA regions (de Queiroz et al. 1995; Swofford et al. 1996). Conflicting histories may be due to the effect of, incomplete or differential sorting of ancestral polymorphisms, horizontal transfer or recent duplications and subsequent extinction of paralogs (Maddison 1989; Reed and Sperling 1999). In our study, we used the best models of evolution as suggested by ModelTest and the separate datasets were found to be congruent by the Partition Homogeneity test. However, the separate datasets for each region individually lacked sufficient sequence variation. This observation of low DNA sequence divergence for the African *Asclepias* complex regardless of the high morphologically diversity has been seen also in the results of Goyder et al., (Goyder et al. 2007) and Fishbein et al (accepted). Such a situation is usually indicative of a recent origin of a group, a phenomenon which usually leads to morphological diversification occurring faster than genetic variation (Baldwin 1997; Bateman 1999; Harris et al. 2000; Malcomber 2002). It seems therefore plausible that the short internal and terminal branches in the African *Asclepias* complex may actually be a reflection of a recent rapid radiation in which species originated almost simultaneously over a short period resulting in the poor DNA sequence divergence exhibited by our data. Such data can hardly be

expected to resolve species relationships e.g. (Baldwin 1997; Bateman 1999; Fishbein et al. 2001; Harris et al. 2000). Since our data exhibits a paucity of sequence variation among taxa the scenario of a recent rapid radiation is a highly plausible explanation for the lack of sufficient basal resolution in the recovered phylogeny. One possible explanation for a rapid morphological divergence and speciation for the African *Asclepias* complex would be the relatively recent formation of suitable habitats for the group on the African continent. Ecologically most species of the African *Asclepias* occur in highland grasslands or open woodlands (Goyder 1998b; Goyder 2001b; Goyder and Nicholas 2001; Kupicha 1984; Langley 1980; Nicholas 1982; Nicholas and Goyder 1992). Such habitats became available in Africa starting from the Miocene when areas previously occupied by forest begun giving way to open savanna and grassland as aridity increased over the continent (Axelrod and Raven 1978; Maley 1991). Such newly formed habitats would have provided opportunities for colonization and rapid morphological adaptation that outstrips genetic diversification (Baldwin 1997; Harris et al. 2000; Malcomber 2002).

Our study adds important pieces to the two earlier attempts by Goyder et al (Goyder et al. 2007) and Fishbein et al.(accepted) to reconstruct the evolutionary history of the African *Asclepias* complex in which they made the initial attempt to resolve phylogenetic relationships for relatively broad samples of the African *Asclepias* generic complex. *Trachycalymma pseudofimbriatum* and *Gomphocarpus* especially the *G. interger* group plus *G. tomentosus* and *G. filiformis* have emerged as vital to understanding the evolution of the Asclepiadinae, confirming at least in part Fishbein's unpublished results [1996, quoted in Goyder and Nicholas (Goyder and

Nicholas 2001)] which suggested that *Gomphocarpus* was key to the understanding of the evolutionary history of the old and new world lineages of the *Asclepias* complex. The resolution of the *G. integer* plus *G. tomentosus* and *G. filiformis* clade (A) which seems to have evolved in Southern Africa as sister to the rest of the African *Asclepias* complex except *T. pseudofimbriatum*, represents a new contribution to the understanding of Asclepiadinae evolution. Species resolved in this clade were not sampled in Goyder et al (Goyder and Nicholas 2001) except for *G. tomentosus*, whose placement was however, not well supported in that study. Even though most genera are not monophyletic as circumscribed, certain strongly supported core lineages of many of the genera still have emerged in this study, to which other unsampled and unresolved species remain to be tested for possible membership.

A note about the SH-Test

Even though the SH tests of generic monophyly did not result in any significant values, it is worth noting that this test has been found to be extremely conservative and sensitive to the number of trees compared due to the confidence intervals that are estimated to account for multiple comparisons (Buckley 2002; Goldman et al. 2000; Strimmer and Rambaut 2002). Furthermore The results of the SH test would therefore best be interpreted as indicating lack of overwhelming evidence for the non monophyly of tested clades rather an suggestion of strong evidence for monophyly. Additionally, other indices of support have strongly indicated the non monophyly of a number of genera.

Core Groups/Lineages within the main African clade (L)

Within the putative *Gomphocarpus*, clade H (Figs. 1 & 2) coincides with a lineage that includes some members of the *G. physocarpus* group (Goyder and Nicholas 2001) plus *G. abbyssinicus* a putative member of the *G. phillipsiae* group (clade HO) and the *G. fruticosus* - *G. physocarpus* group (clade HI). The remnant *Gomphocarpus* should be built around this clade. Another core *Gomphocarpus* lineage whose relationship to the rest of the taxa remains to be determined is the clade T lineage corresponding with the all members of the *G. glaucophyllus* group, consisting of montane grasslands and the *Brachystegia* belt clade that spans south central Africa. This clade is characterized by unbranched annual stems and thickened non-tuberous, but woody rootstocks (Goyder and Nicholas 2001).

The *Stathmostelma* clade (B) forms a lineage of seasonally waterlogged grassland species, occurring in drier parts of eastern and central tropical Africa that includes four species one of them being the type species *S. gigantiflorum*. This therefore forms the core *Stathmostelma* clade but it is not clear whether *S. spectabile* is related to this clade.

The core *Xysmalobium* lineage is formed by clade F since it contains the type species *X. undulatum*. This group appears to have evolved in the tropical latitudes except for *X. undulatum* which is only known from South Africa (Langley 1980).

The genus *Aspidoglossum* may be paraphyletic since species of *Schizoglossum* may be nested within the *Aspidoglossum-Schizoglossum* core clade (I). The close relationship of *Aspidoglossum* and *Schizoglosum* is also supported by results of

Goyder et al (Goyder et al. 2007). However owing to weak MP bootstrap support, confident interpretation of relationships for the two genera will still have to await further analysis.

Pachycarpus clades G and the SI form two core lineages whose relationship still remain unclear whereas all the *Glossostelma* species are strongly supported as monophyletic. Other small strongly supported lineages include the *A. aurea* and *A. cucullatum* lineage and a lineage of *A. randii* plus an indeterminate species (*Richards 22589*). There is basically no disagreement in strongly supported parts of the phylogeny between this study and the Goyder et al study (Goyder et al. 2007).

The resolved lineages within the African *Asclepias* will be useful in testing hypotheses regarding evolution of phenomena such as plant-pollinator interactions, plant defense traits and morphological adaptation to different ecological habitats within the African *Asclepias* complex. Our findings will facilitate comparisons of such evolutionary trends with the related and well studied American *Asclepias* sensu stricto.

However, more phylogenetic studies should be conducted to obtain a more comprehensive and strongly supported phylogenetic picture and such future studies need not only include extended sampling of loci (which will enable greater resolution and clade support) but also more species to include a greater representation of African Asclepiadinae (which will help consolidate decisions of generic boundaries). The genera *Woodia* Schltr., *Cordylogyne* E.Mey., *Fanninia* Harv. and *Periglossum* Decne. were not sampled in this study and even though they were sampled by Goyder et al (Goyder and Nicholas 2001), they were either unresolved or their placements were not

supported. They must therefore be included in future studies to confidently establish their relationships. *Periglossum* Decne. has not been sampled here or by Goyder et al (Goyder and Nicholas 2001) and should also be included in future efforts.

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TABLE 1. Taxonomic history of the African *Asclepias* generic complex. Genera without author names were described by the respective authors on top of each column

E MEYER 1838	**SCHUMMAN** (1893-1897)	**N.E. BROWN** (1902-1909	**BULLOCK** 1952-1963	**GOYDER** (1992 - to date)
	Asclepias L.		*Asclepias* L. .	*Asclepias* L.
	Gomphocarpus R.Br.	*Asclepias* L. .	*Gomphocarpus* R.Br.	*Gomphocarpus* R.Br.
Xysmalobium R.Br.	*Xysmalobium* R.Br.	*Xysmalobium* R.Br.	*Xysmalobium* R.Br.	*Xysmalobium* R.Br.
Aspidoglossum E. Mey			*Aspidoglossum* E. Mey	*Aspidoglossum* E. Mey
Cordylogyne E. Mey	*Cordylogyne* E. Mey	*Cordylogyne* E. Mey		*Cordylogyne* E. Mey
Lagarinthus E. Mey			*Lagarinthus* E. Mey	*Asclepias* L. .
Pachycarpus E. Mey	*Gomphocarpus* (Section *Pachycarpus*)	*Pachycarpus* E. Mey	*Pachycarpus* E. Mey	*Pachycarpus* E. Mey
Parapodium E. Mey	*Parapodium* E. Mey	*Parapodium* E. Mey		*Parapodium* E. Mey
Schizoglossum E. Mey	*Schizoglossum* E. Mey	*Schizoglossum* E. Mey		*Schizoglossum* E. Mey
		Periglossum Decne		*Periglossum* Decne
	Fanninia Harv.	*Fanninia* Harv.		*Fanninia* Harv.
	Margaretta		*Margaretta*	*Margaretta*
		Woodia Schltr.		
			Glossostelma	*Glossostelma*
	Stenostelma Schltr.		*Stenostelma* Schltr.	*Stenostelma* Schltr.
	Stathmostelma K. Schum	*Stathmostelma* K. Schum	*Stathmostelma* K. Schum	*Stathmostelma* K. Schum
				Miraglossum Kupicha
				Aspidonepsis A. Nicholas & D.J. Goyder
			Trachycalymma Bullock	*Trachycalymma* Bullock

TABLE 2. Primers employed in DNA amplification and sequencing.

Region	Primer Name	Primer sequence	Putative utility	Source
rpl16 intron				
	rpl16-F71	GCT ATG CTT AGT GTG TGA CTC GTT G	Universal	(Small et al. 1998)
	rpl16-R1661	CGT ACC CAT ATT TTT CCA CCA CGA C	Universal	(Small et al. 1998)
	rpl16-F608	GAT TCA CTG GTC GGG ATG GCG A	Asclepias	Agrawal & Fishbein 2008
	rpl16-R697	GTT TTC GCG GGC GAA TAT TTA CTC	Asclepias	Agrawal & Fishbein 2008
trnC-rpoB spacer				
	rpoB 5'R	GTA GAT ATT CCC TCA TTT CC	Universal	(Ohsako and Ohnishi 2000)
	trnC 5'R	TGC CTT ACC ACT CGG CCA T	Universal	(Ohsako and Ohnishi 2000)
	trnC-431F	AGA ACG CAA CCC GCG CTG C	Asclepias	Agrawal & Fishbein 2008
	trnC-759R	CCA ATC CGT TTG AAT ACC CGA	Asclepias	Agrawal & Fishbein 2008
trnS-G spacer + intron				
	trnG5'2G	GCG GGT ATA GTT TAG TGG TAA AA	Universal	(Shaw et al. 2005b)
	trnG5'2S-DC	CAA ACC GAA AAC AMC GAC CC	Asclepias	this study
	trnG-DC	GGG GTT ATA GTA GAC GTC GA	Asclepias	this study
	trnS-DC	CCA CTC AGC CAT CTC TCC TAA	Asclepias	this study

TABLE 3. Summary statistics of the aligned datasets of three chloroplast regions (*trnS-trnG* intergenic spacer/*trnG* intron, *trnC-rpoB* intergenic spacer, and *rpl16* intron) for 86 taxa of the African *Asclepias* complex and outgroups.

	trnS-trnG /trnG	***trnC-rpoB***	***rpl16* intron**	**Combined**
Total length of aligned sequences	1388	1475	1499	4362
Number of indels coded	1	8	3	12
Length with recoded indel positions deleted	1382	1372	1473	4227
Missing data	1.2%	1.2%	1.2%	1.2%
Parsimony informative characters with original indel positions deleted after coding and indels included	62 (4.5%)	80 (5.8%)	70 (4.8%)	188 (4.5%)
Number of ambiguously aligned sequence positions that were omitted from analysis	51	26	170	247
Taxa missing sequence	*Asclepias disparilis*	*Trachycalymma buchwaldii*	*Asclepias disparilis* Goyder 4895	–

TABLE 4. Results of the Shimodaira-Hasegawa tests of monophyly conducted in PAUP* for eight hypotheses. These hypotheses were evaluated at $\alpha = 0.05$ against a null distribution based on 10000 replicates analyzed with the RELL method. All *P*-values were non-significant.

Null Hypothesis tested	**-ln L**	**Diff -ln L with Best Tree**	***P*-value**
ML Tree	10723.95794	-	-
Monophyly of *Aspidoglossum*	10759.04404	35.08610	0.2285
Monophyly of African *Asclepias* sensu lato including *Trachycalymma pseudofimbriatum*	10731.08391	7.12597	0.7764
Monophyly of *Gomphocarpus*	10759.87977	35.92183	0.2450
Monophyly of *Pachycarpus*	10752.48461	28.52667	0.3434
Monophyly of *Schizoglossum*	10759.60022	35.64228	0.2502
Monophyly of *Stathmostelma*	10743.95368	19.99574	0.5419
Monophyly of *Xysmalobium*	10739.48459	15.52665	0.6090

FIG. 1: Maximum parsimony strict consensus tree of the African *Asclepias* generic complex based on *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA data. Numbers on the left are bootstrap values and numbers on the right are Bayesian posterior probabilities. Letters represent the nodes above them and their respective clades. Thick branches represent nodes with both MP bootstrap and Posterior probability of at least 75 and 100 respectively.

ntosus

FIG. 2: Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method and based on *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA data. Letters represent the nodes and their respective clades.

CHAPTER 2: EARLY LINEAGE DIVERGENCE WITHIN THE ASCLEPIADINAE (APOCYNACEAE) INFERRED FROM NUCLEAR *PGIC* AND CHLOROPLAST DNA

Early lineage divergence within the Asclepiadinae (Apocynaceae) inferred from nuclear *PgiC* and chloroplast DNA

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ABSTRACT

Previous phylogenetic analyses of the African Asclepiadinae have resolved a number of relationships for the African Asclepias generic complex but only provided weakly supported resolution for relationships of few terminal clades leaving the early diverging lineages poorly understood. These relationships have mainly been inferred from chloroplast sequences of *trnT-L*, *trnL-F* spacers, *trnL* intron, *rpl16* intron, *trnC-rpoB* and *trnS-G* spacer, *trnG* intron and the nuclear ITS. This study utilizes the partial *PgiC* gene region in combination with previously used cpDNA sequences to achieve more resolution and support for the early divergent lineages of the Asclepiadinae phylogeny and to determine whether the nuclear *PgiC* gene sequences support core clades and relationships resolved by previous studies. DNA sequences were obtained for 51 species representing 11 genera and analyzed using maximum parsimony, maximum likelihood and Bayesian phylogenetic methods. These analyses have resolved early diverging lineages within the African Asclepiadinae and supported the monophyly of the *Asclepias* generic complex. The Ethiopian highlands endemic species *Trachycalymma pseudofimbriatum* forms the earliest diverging lineage of the African *Asclepias* generic complex and a lineage including the clade of narrow leaved species of the *Gomphocarpus* integer group plus *G. filiformis* is the next diverging lineage.

Keywords – African Asclepiadinae, *Asclepias*, milkweed, *PgiC*, nuclear DNA

INTRODUCTION

The Tribe Asclepiadinae (Apocynaceae) consists of plants with a disjunct distribution in the Americas (mostly North America) and the old world (Asia and Africa) with the American species all belonging to one genus *Asclepias* L. sensu stricto. In the old world group of the Asclepiadinae, three genera (*Pergularia* L., *Calotropis* R. Br. and *Kanahia* R. Br.) are early diverging lineages relative to both the rest of the old world Asclepiadinae which have been referred to as African Asclepias complex (Goyder et al. 2007) and the American *Asclepias* s.s. The African *Asclepias* complex (Asclepiadinae, Apocynaceae) consists of plants native to the Old World and whose diversity is centered in southern and eastern Africa (Liede 1997). Their cumulative range covers the area from Sinai and the Dead Sea in the north through the Arabian Peninsula and most of Africa to the Cape region in the south. This group is absent from Madagascar. These plants include approximately 250 species in 17 of the 21 genera in the subtribe Asclepiadinae (Goyder 2001a) within Apocynaceae (Endress and Bruyns 2000; Liede 1997).

Generic delimitations in the African Asclepiadinae have been problematic. Previously phylogenetic relationships were determined based on plastid DNA data (Chapter 1, Goyder et al. 2007, Fishbein et al. accepted), in an effort to determine species affinities and test generic circumscriptions. These analyses demonstrated that individual gene regions produced poorly resolved phylogenies, whereas use of multiple regions progressively improved resolution among close outgroups in

Asclepiadinae and in terminal, but not in the early diverging lineages, within the African *Asclepias* complex (Goyder et al. 2007, Fishbein et al., Chapter 1).

The use of plastid DNA simplifies analyses because it is haploid (and thus does not exhibit allelic variation) and shows reduced intraspecific variation relative to nuclear loci (Small et al. 2004). However, there are necessary considerations for interpreting a plastid DNA based phylogeny. Since many plants hybridize and undergo introgression, cpDNA variation may reflect the history of introgressive transmission in addition to phylogenetic divergence (Rieseberg and Soltis 1991, Rieseberg and Wendel 1993; Small et al. 2004). In the absence of knowledge about past hybridization, phylogenies may contain erroneous placements of such species in clades with their maternal parents (in most Angiosperms) (Small et al. 2004).

To add more informative sites to the chloroplast data and to counter the effect of potentially misleading phylogenetic inference from a single locus, such as cpDNA, nuclear ribosomal DNA has been generally employed as an additional and independent source of data for estimating plant phylogenies (e.g. Gielly et al. 1996; Hoot and Taylor 2001; Rieseberg et al. 1990). Within Asclepiadinae, addition of the nuclear internal transcribed spacer region (ITS) to the chloroplast (*trnT-L* and *trnL-F* spacers *trnL* intron) data (Goyder et al. 2007) helped to resolve some relationships but still fell short of achieving strong support or confident resolution of particularly some early diverging relationships. The more recent chloroplast based phylogenetic study detected previously unresolved clades and a few interesting basal relationships (chapter 1). That analysis (chapter 1) however, still produced a number of polytomies and some weakly supported relationships. The African *Asclepias* complex was

resolved in two lineages, one containing only *Trachycalymma pseudofimbriatum* and the other containing all other African *Asclepias* sensu lato. These two lineages were resolved as part of a polytomy with the American *Asclepias* sensu stricto clade. The large African clade was composed of two sister lineages, one containing all sampled members of the *Gomphocarpus integer* group plus *G. tomentosus* and *G. filiformis* and the other containing the rest of the African species. In the major African clade, many genera were found to be non monophyletic namely *Gomphocarpus*, *Pachycarpus*, *Stathmostelma*, *Xysmalobium*, *Trachycalymma*, *Schizoglossum* and *Aspidoglossum*. However, *Glossostelma* was resolved as monophyletic. Species still classified as *Asclepias* sensu lato showed various, strongly supported affinities. *A. aurea* (Schltr.) Schltr. was sister to *A. cucullata* Schltr., *A. randii* S. Moore was sister to a sample attributable to *Gen. indet. aff. Asclepias* (10917), *A. macropus* (Schltr.) Schltr. was sister to *A. praemorsa* Schltr., *A. densiflora* was sister to *Xysmalobium aceratoides* (Schltr.) N.E. Br., and *A. gibba* Schltr. was sister to *A. disparilis* N.E. Br. Either, more information is required to resolve the polytomies in the cpDNA analysis (chapter 1), or these may be hard polytomies representing several simultaneous lineage divergence events, which have left no molecular evidence of any common ancestral lineage.

Introns of low copy nuclear gene sequences seem appropriate for augmenting results of cpDNA studies as they have been shown to offer variable and phylogenetically informative molecular markers for plant groups in which plastid DNA has produced only very limited phylogenetic resolution, such as at low taxonomic levels or in rapidly diversifying lineages (e.g. (Small et al. 2004), (Mort

and Crawford 2004) (Gaut 1998). Low copy nuclear genes also exhibit biparental inheritance and can therefore potentially reveal reticulate ancestry (Small et al. 1998).

In this study, partial sequences of the low copy nuclear gene *PgiC*, which encodes the cytosolic isozyme phosphoglucose isomerase, were obtained to achieve more resolution and stronger support in the African *Asclepias* generic complex. The *PgiC* gene possesses 23 exons that are interspaced by 22 introns (Thomas et al. 1992; Thomas et al. 1993). This gene has previously been used successfully to explore the phylogenetic relationships of a number of plant groups e.g. tribes of Onagraceae using the *PgiC* region between exons 5 and 21 (Ford and Gottlieb 2007; Ishiyama et al. 2008) and *Stephanomeria* (Compositae) using the region between exons 11 and 21 (Ford et al. 2006). Initial sequencing efforts also indicated a high level of variation among both American and African Asclepiadinae (Fishbein, et al., unpublished data).

The first goal of this study was to utilize the partial *PgiC* gene region to try and achieve more resolution and support for the early divergent lineages of the Asclepiadinae phylogeny. The second goal was to determine whether the nuclear *PgiC* gene sequences could corroborate some of the core clades and relationships resolved by chloroplast DNA inferences or reveal discrepancies between the cpDNA and low copy nuclear *PgiC*, which would be consistent with a history of reticulate evolution.

MATERIALS AND METHODS

Taxon Sampling

DNA sequences of 51 species were sampled representing 11 genera within the African *Asclepias* generic complex and five outgroup genera (See Appendix A). DNA samples were obtained from banks at the Royal Botanic Gardens-Kew, United Kingdom (38 samples) and South African National Botanical Institute-Cape Town, R.S.A. (3 samples) and 10 samples were obtained from additional field and herbarium collections (Fishbein et al. accepted). Eight species of American *Asclepias* s.s. representing seven of 13 major clades (Fishbein et al. accepted) and one species each of the remaining genera of Asclepiadinae, *Pergularia, Kanahia* and *Calotropis*, were utilized as outgroups (Goyder 2001a; Rapini et al. 2003), Fishbein et al., accepted). A more distant outgroup *Cynanchum ligulatum* (Benth.) Woodson from another subtribe, Cynanchinae K. Schum, was also included to root the phylogeny of Asclepiadinae. Chloroplast DNA sequences for 48 of the species sampled were already available for three cpDNA regions (Fishbein et al accepted; Chuba et al in preparation). Chloroplast sequences (*rpl16* intron, *trnC-rpoB* spacer and *trnS-G* spacer/*trnG* intron) for *Miraglossum verticillare* (Schltr.) Kupicha, *Miraglossum pilosum* (Schltr.) Kupicha, *Asclepias crispa* P.J.Bergius and *Stenostelma capense* Schltr were newly obtained.

DNA isolation, amplification and Sequencing

DNA was extracted using the Qiagen DNeasy Plant Mini kit (PE Biosystems). Polymerase chain reaction (PCR) was utilized to obtain sequencing templates for the nuclear *PgiC* (Thomas et al. 1992; Thomas et al. 1993) and three cpDNA regions

(*rpl16* intron, *rpoB-trnC*^{GCA} spacer, and *trnS*^{GCU}-*trnG*^{UUC} spacer/*trnG* intron) (Shaw et al. 2005b; Shaw et al. 2007; Small et al. 1998). The various primers employed in amplifying these chloroplast regions are the same as in Chuba et al. (in preparation). For *PgiC*, the region amplified corresponded to the partial region (730-825 bp of utilized sequences) from exons 11 to 16 of the *PgiC* gene in *Arabidopsis thaliana* (Kawabe and Miyashita, 2000) encompassing four exons and five introns.

Specifically designed primers (Asc11F, Asc14R, Asc13F and Asc16R; Table 5) were used to both amplify and sequence all samples from species within Asclepiadinae. These locus-specific primers were developed for Asclepiadinae in the Fishbein lab at Portland State University (Fishbein et al, unpublished data) permitting the direct sequencing of PCR products, after a series of cloning experiments based on universal primers (Ford and Gottlieb 2007). For some species, primers Asc11F and Asc16R were used to amplify the whole region but in samples that were difficult to amplify, Asc11F+Asc14R and Asc13F+Asc16R pairs were both used to amplify the region in parts. Sequencing was done using all the four primers. In preliminary analyses, there was no evidence of sequence heterogeneity among the clones, giving confidence that sequences obtained from these primers were likely to yield orthologous sequences for the rest of the Asclepiadinae. The *PgiC* sequences from *Cynanchum ligulatum* was only successfully amplified with the degenerate ''universal'' primers AA16R (Ford and Gottlieb 2007) and the specifically designed Asclepiadinae primer Asc11F.

The thermal profile for PCR amplification of the *PgiC* region from genomic DNA was as follows: initial denaturation at 95° C for 2 min, followed by 35 cycles of

95° C for 1 min, 54° C for 1 min, 72° C for 2 min, and a final extension at 72° C for 7 min. In some cases the entire region could not be amplified, in which case they were amplified in two parts using combinations of external and internal primers as follows: Asc16R with Asc13F and Asc11F with Asc14R. In the case of the Asc16R with Asc13F combination the same thermal profile was utilized but with annealing temperature increased to 56° C. The *trnS-G* region was amplified using the same thermal profile as in Chuba et al. (in preparation). Amplification conditions for the chloroplast regions were as in Chuba et al (in preparation). Conditions for the *PgiC* region generally consisted of 50 µl reactions with approximately 2µL (~20 - 40ng) of DNA, primer pair containing 1 µM of each primer, 0.2 mM dNTPs and a combination of 1× PCR reaction buffer plus 0.2 U HotMaster Taq DNA polymerase obtained from Eppendorf North America Inc. (Westbury, NY USA). Five percent Dimethyl sulfoxide (DMSO) was also added to all PCR reactions to mitigate the effects of secondary structures formation in the *PgiC* region (Bachmann et al. 1990; Zhang et al. 1992). The templates were sequenced using the ABI BigDye® Terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc. California). The general cycle sequencing profile was as follows: initial denaturation at 80° C for 4 min, followed by 25 cycles of 96° C for 10 sec, 52° C for 5 sec. and 72° C for 4 min. Unincorporated dye terminators were removed using Sephadex G-50 columns. The cleaned sequences were then analyzed using an ABI 3700 Genetic analyzer sequencer (Oregon Health Sciences University Vollum Lab).

Alignment and phylogenetic Analysis

DNA sequences were assembled using SeqMan™ II (DNASTAR 1999), and manually aligned using the Se-Al v2.0 (Rambaut 2007) in combination with MacClade (Maddison and Maddison 1992). Appropriate nucleotide substitution models for each region and the combined dataset were determined using ModelTest 3.7 (Posada and Crandall 1998).

Before combining the chloroplast and the *PgiC* data sets, a partition homogeneity test (incongruence length difference, ILD) (Farris et al. 1994) was performed on the matrices in PAUP*v4.0b10 (Swofford 2001) to determine the level of congruence between them. The partition homogeneity test has been subject of many discussions supporting or disputing its usefulness as a means of determining whether data sets should be analyzed in combination or not. Some studies that have compared the partition homogeneity test with other tests have concluded that the partition homogeneity test is the best method for this purpose (Cunningham 1997) whereas others have argued that the partition homogeneity test must never be used as a test of data combinability (Yoder et al. 2001). The test has been shown to be highly susceptible to type I error (Hipp et al. 2004) because of its sensitivity to between partition differences in noise and rate heterogeneity among sites across the data partitions (Darlu and Lecointre 2002; Yoder et al. 2001). Others have suggested that the partition homogeneity test should only be used as a starting point in testing data partition congruence and that other criteria should be used to determine the nature of incongruence (Hipp et al. 2004; Mason-Gamer and Kellogg 1996).

In this study the partition homogeneity test was initially used to determine congruence between the nuclear and chloroplast DNA regions and this was followed by a node to comparison of trees estimated from separate chloroplast and nuclear regions {Reeves, 2001 #1914}. A number of taxa were incongruently placed between the cpDNA and *PgiC* phylogenenies. Therefore, the partition homogeneity test was also conducted on datasets with taxa that had discordant placement between the nuclear and chloroplast datasets, individually and successively removed before conducting the test.

The partition homogeneity test rejected the null hypothesis of homogeneity of the phylogenetic signal between the cpDNA and the *PgiC* region ($p = 0.01$) with all the taxa included and with taxa having incongruent placement between the chloroplast and *PgiC* datasets excluded. However, when the cpDNA topology (Chapter 1) was compared to the *PgiC* topology on a node-by-node basis, an approach deemed to give a better assessment of homogeneity of the phylogenetic signal than the occasionally unreliable partition homogeneity test (Reeves et al. 2001; Yoder et al. 2001), there was no highly supported topological incongruence (>75 BP) except for the placement of *Asclepias aurea*, *Asclepias cucullata* and the outgroup taxon *Pergularia daemia*, (which was surprisingly placed as sister to two South American species *Asclepias barjoniifolia* and *A. candida*).

According to Huelsenbeck (Huelsenbeck et al. 1996) and Wiens (Wiens 1998), even if the partition homogeneity is rejected, more accurate phylogenetic estimates can be obtained by combining data sets that individually produce topologies that are are mostly congruent. Even though parts of the combined tree can potentially be

affected by the difference in the phylogenetic histories of individual datasets, the overall accuracy can be increased by the increased number of characters applied to the parts of the tree that are congruent (Wiens 1998).

The two datasets were therefore combined due to the high level of congruence in most parts of the topology. However, tree searches were conducted initially with all taxa included followed by analyses with taxa having conflicting placement between the *PgiC* and cpDNA (Chapter 1) excluded from the combined analyses. The phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) methods in PAUP*v.4.0b10 (Swofford 2001). For parsimony analysis potentially informative insertion or deletion events (indels) were coded and used in the analysis. Indels were recoded as binary or multistate characters with the sequence data in the indel regions excluded from the matrix. A total of 17 indels were coded of which nine where in the chloroplast data sets and eight were in the *PgiC* region. All parsimony analyses were conducted using a heuristic search with tree bisection and reconnection (TBR) branch swapping, 10,000 addition sequence replicates, 10 trees held at each step and no more than 100 trees saved in each replicate. A parsimony ratchet analysis (Nixon 1999) was conducted using the PAUPRat program (Sikes and Lewis 2001) in combination with PAUP* in order to further search for most parsimonious trees (MPTs). Characters re-weighted per iteration were maintained at the default setting of 25%. A total of 201 trees were saved for each of the 20 ratchet replicates. These were filtered to retain only the best trees, and then summarized by computing a strict consensus tree. Support for each node of the inferred phylogeny was estimated by parsimony bootstrap analysis

(Felsenstein 1985) (5000 bootstrap replicates) using a heuristic search with TBR branch swapping, 10 random addition sequences per bootstrap replicate, 1 tree held at each step during each random addition sequence and 10 trees saved per replicate. A BS value of 75% or greater was considered good support whereas a bootstrap of 65–74% was designated moderate support. A less than 65% BS was considered weak (Meerow and Clayton 2004). Bremer support (decay indices, DI) was also computed using PRAP 2 (Muller 2007). One hundred heuristic searches with random addition sequence were implemented for each constraint statement postulated by PRAP2 (Muller 2007), saving no more than 10 trees per search. A decay Index (DI) =2 or more was considered good support for a clade (Meerow and Clayton 2004). Consistency index CI (Kluge and Farris 1969) and rescaled consistency index RC (Farris 1989) were calculated separately for the chloroplast and nuclear regions to assess homoplasy in each of the datasets.

The maximum likelihood ratchet (Morrison 2007) as implemented in PRAP2 (Muller 2007) was used to conduct a Maximum likelihood (ML) analysis. The ratchet algorithm is a tree search strategy that involves the following steps; first generating a starting tree, followed by initial branch swapping under original character weights, random selection of a subset of characters and giving them additional weight each, performing branch swapping on the ML tree from the previous step using the reweighted matrix, setting all weights for the characters back to the "original" weights and performing branch swapping on the ML tree from the previous step and finally returning to the second step. At each step one (or few) trees are held and used as starting trees for the next step. Several of such iterations (e.g 200 in this study) are

perfomed. The ML ratchet analyses were conducted under the GTR+I+G nucleotide substitution model as suggested by the hierarchical likelihood ratio tests (hLRT) and Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Crandall 1998) for both the combined and nuclear data sets. A total of 25% characters were upweighted and only one tree was saved per iteration. Ten ratchet iterations were performed.

Bayesian analysis was applied using MrBayes version 3.2.1 (Huelsenbeck and Ronquist 2001) to the *PgiC* data alone and the combined chloroplast and *PgiC* matrix respectively to approximate posterior probability support for the MP and maximum likelihood estimates of the phylogenetic relationships.

The combined chloroplast and nuclear dataset was partitioned by gene region and the best nucleotide substitution model, GTR+I+ G, applied to each of the three data partitions in the chloroplast dataset and to the *PgiC* partition, this being the model suggested by the Akaike information criterion (AIC) test (Akaike 1974) in Modeltest 3.7 (Posada and Crandall 1998). Seven chains (one cold and six heated) were used and run for a total of 11,000,000 generations, utilizing the program's default priors on model parameters and branch lengths and sampling trees from the cold chain every 1000 generations. Two Markov chain Bayesian analyses were performed simultaneously. Stationarity and convergence of model parameters were evaluated using Tracer version 1.4 (Rambaut and Drummond 2007). The 'burn in' was determined in Tracer version1.4 (Rambaut and Drummond 2007) and trees in the 'burn in' phase discarded in each run so that approximately only trees in the stationary phase of the chain were considered. A 50% majority-rule consensus of the remaining

trees was then computed to determine Bayesian posterior probabilities (BPP) as clade support for the inferred MP and ML phylogeny. The combined MP and ML analyses were perfomed with all species included and with species that had discordant placement between the separate nuclear and chloroplast analyses excluded. Maximum likelihood bootstrap support was also estimated for both the *PgiC* only and the combined analysis of chloroplast and nuclear DNA phylogenies, using RAxML v. 7.0.4 (Stamatakis et al. 2008) under the GTR+G+I model of sequence evolution with the number of bootstrap replicates estimated by the program.

RESULTS

The aligned *PgiC* sequences resulted in 1372 characters for 51 taxa (Table 6). Of these, 45 positions could not easily be aligned and were consequently excluded from all the analyses. Combined aligned sequences of chloroplast and nuclear sequences resulted in 5585 characters for 51 taxa (Table 6). Nine percent (123) of the 1339 included characters being parsimony informative. The MP analysis of the nuclear *PgiC* dataset alone with all species included yielded 38640 shortest trees (L= 583 steps, CI excluding uninformative characters = 0.5815, RI = 0.7551, RC = 0.5855). The strict consensus of these MPTs is presented in Fig. 3. This tree had little resolution especially at the base where it was essentially a polytomy (Fig. 3). The ML analysis of the *PgiC* dataset resulted in the tree presented in Fig. 4

In both the MP and ML trees (Figs. 3 and 4), the Old World outgroup species *Pergularia deamia* was surprisingly placed inside the well supported (BS 92, PP 100) American *Asclepias* s.s. clade (Figs. 3 and 4, Clade NA). Within this clade it was

strongly supported (BS 90, PP 97) as sister to the South American species *Asclepias barjioniaefolia* and *Asclepias candida*. This placement contradicts all previous phylogenetic studies and may only be as a result of sequencing error as explained below, therefore requiring further confirmation. *Gomphocarpus praticolus* was resolved as sister to the clade of American species and *P. daemia* (NA) without any MP bootstrap or Bayesian support. Among the African species a few clades were resolved (Fig. 3), with some of them corroborating results of the chloroplast data (Chapter 1) and a few others showing previously unsupported relationships. *Asclepias aurea* and *Asclepias gibba* were strongly supported (Figs. 3 and 4, Clade NB: BS 86, PP 100) as sister by the *PgiC* data, a relationship which was not resolved in the cpDNA data alone (Chapter 1). A clade that included two subspecies of *G. fruticosus* and *G. physocarpus* (Figs. 3 and 4, Clade NC: BS 90, PP 100) was also resolved in the analysis of cpDNA alone (Chapter 1). However the *PgiC* data placed this clade with *G. abbyssinicus* and *Asclepias macropus* in a more inclusive clade (ND, Figs. 3 and 4), where as the cpDNA analysis (Chapter 1) placed *A. macropus* with strong support as sister to *A. praemorsa*. However the clade placement for the *A. praemorsa-A.macropus* sister pair in the cpDNA analysis (Chapter 1) had no bootstrap support and very weak Bayesian posterior probability support. The MP analysis of *PgiC* data alone did not resolve the position of *A. praemorsa* (Fig. 3) whereas the ML analysis resolved it as sister to a clade (NH, Fig. 4), which contains *Asclepias macropus*. However this placement had weak support (BPP 82). Another moderately supported clade recovered by the *PgiC* data was the *Gomphocarpus-Pachycarpus* clade (Figs. 3 and 4; NE: BS 73, PP 90) consisting of two sister clades of *Gomphocarpus* (Clade NF:

BS 96, PP 100) and *Pachycarpus* (Clade NG: BS 74, PP 99) species. The ML tree (fig. 4) had a number of other additional clades that were resolved but with very weak or no support. These include a clade NI (fig. 4) which contains the *Gomphocarpus pachycarpus* clade NE, *Trachycalymma pseudofimbriatum*, *Glossostelma spathulatum* and *Trachycalymma buchwaldii*. The *Asclepias gibba* - *Asclepias aurea* clade (Clade NB, fig. 4) was resolved with no support as sister to the *Gomphocarpus cancellatus* – *Asclepias crispa* clade. The members of the informal *Gomphocarpus integer* group {Goyder, 2001 #663} were resolved with *Gomphocarpus filiformis* in one weakly suppoted (BPP 59) clade (NI), which was also resolved by the chloroplast analysis (Chapter 1). Species of *Miraglossum*, *Schizoglossum* and *Aspidoglossum* (except *A. heterophyllum* and *A. interruptum*), genera that have been considered as closely related {Kupicha, 1984 #746}, were resolved in a weakly supported (BPP 73) clade (NJ) with *Asclepias randii*. *Stenostelma capense*, *Margaretta rosea* and *Stathmostelma pedunculatum*. The rest of the relationships resolved in the ML analysis of *PgiC* (Fig. 4) were not support by Bayesian posterior probabilities.

The combined sequences resulted in an aligned dataset of 5584 characters. Of the 784 (15%) variable characters, after exclusion of ambiguously aligned characters, 259 (4.8%) were parsimony informative. The combined MP analysis of the nuclear and cpDNA dataset yielded 150 shortest trees (L= 1191 steps, CI excluding uninformative characters = 0.5615, Retention index (RI) = 0.7102, RI= 0.7825) and the strict consensus of the MPTs is presented in fig. 5. The parsimony ratchet analysis also yielded shortest trees of the same length (1191 steps) and the same consensus tree.

The ML tree recovered from the maximum likelihood ratchet analysis of the combined cpDNA and *PgiC* dataset (-ln likelihood of 15245.28350) is presented in Fig. 6. The Bayesian analysis of combined data did not show attainment of close convergence of the two runs as indicated by standard deviation of split frequencies (> 0.01), but all model parameters appeared to attain or closely approached stationarity as indicated by their examination using Tracer, which also suggested a burn in phase of 1,100,000 generations. Therefore the first 11,000 parameter and tree samples were discarded. The effective sample sizes for most parameters were sufficiently high (above 100), indicating adequate sampling from the posterior probability space. The potential scale reduction factor values for all parameters were 1.000 or very close to this value, indicating possible convergence of the two runs. Therefore the post burn-in trees were combined into a single pool of 2,178,000, which was used to calculate the posterior probabilities for clades as indicated on the MP (Fig. 5). The topology recovered by the ML analysis had an overall structure similar to the MP tree.

The combined MP and ML analyses of cpDNA and *PgiC* resulted in a topology (Figs. 5 and 6) that greatly increased basal resolution and support for some of the clades recovered in the cpDNA alone. The MP analysis resolves both the African *Asclepias* complex (Clade C4: BS 72, PP 100) and the American *Asclepias* sensu stricto clade (Clade C1: BS 95, PP 100, DI 7) as monophyletic sister clades. This rsolution is also supported by the ML analysis (Fig 6). Further analysis of the combined data with taxa having conflicting placements between nuclear and chloroplast analyses excluded, also resolved the African *Asclepias* complex as monophyletic (Fig. 7).

Furthermore *Trachycalymma pseudofimbriatum* Goyder has been resolved as sister to the rest of the African *Asclepias* sensu lato (clade C5; Fig. 5: BS 82, PP 100, DI 2), a placement that was not resolved in the analysis of the cpDNA dataset alone (Chapter 1, Fishbein et al. in press) or cpDNA and ITS sequence data (Goyder et al. 2007). This resolution of T. pseudofimbriatum is strongly supported by ML analysis (Fig. 6). The large clade that is sister to *Trachycalymma pseudofimbriatum* has two sister lineages, a smaller one (Clade C18; Fig. 5, BS 82, PP 100 DI 1), consisting of species of the *Gomphocarpus integer* (N.E. Br.) Bullock group (Goyder and Nicholas 2001) plus *G. filiformis*, and a weakly supported clade containing the rest of the African *Asclepias* sensu lato (Fig. 5, clade C6;: BS 57, PP 99, DI 1; Fig 6. ML BS 67). The ML tree of the combined cpDNA and *PgiC* datasets (Fig.7) was exactly the same as the MP tree in the remaining parts of the topology and these are discussed below.

DISCUSSION

This study has resolved early diverging lineages within the African Asclepiadinae, an important contribution to understanding the evolution of the Asclepiadinae. The results of this study corroborate findings of previous analyses based on chloroplast DNA data (Chapter 1) as elaborated below. However, this study shows some conflicting phylogenetic histories. In particular, the data show conflicting phylogenetic histories between the cpDNA and *PgiC* gene data in the strongly supported (BS 92, PP 100) placement of the Old World outgroup *Pergularia daemia*

within the American *Asclepias* s.s. clade by the nuclear *PgiC* region, as sister to the South American clade of *A. candida* and *A. barjoniaefolia* (BS 90, PP 97). Such a resolution could be a possible effect of factors such as sampling or analytical error (Hipp et al. 2004), incomplete or differential sorting (deep coalescence) of ancestral alleles (de Queiroz et al. 1995; Swofford et al. 1996) (Rieseberg and Wendel 1993) or horizontal transfer (Avise 2000; Broyles 2002; Maddison 1989; Reed and Sperling 1999). . Even though interspecific hybridization has been documented in Asclepiadinae (Broyles 2002; Broyles et al. 1994; Hatfield and Kephart 2003; Kephart et al. 1988), the current geographic scenario does not seem to favor horizontal transfer as a possible explanation for the placement of *P. daemia.* Also if deep coalescence was involved, ancestral polymorphisms persisting through so many nodes of the tree should result in many more instances of discordant placements of species of the North American clade in which the South American *A. candida* and *A. barjoniaefolia* are nested (Fishbein et al accepted). Another possible explanation would therefore be that placement is an artifact of long branch attraction since *Pergularia daemia* and the *A. candida* and *A barjioniaefolia* clade have relatively fast evolving *PgiC* sequences (Fig 4). A further possible explanation would be sequencing error or mix up of DNA samples. This should preferably be checked after a resequencing of a newly obtained sample of DNA. However, based on an examination of the alignment, the *Pergularia* sequence did not resemble any of the other sequenced species.

The African *Asclepias* generic complex (clade C4, Fig. 5) was resolved as monophyletic (DI 1, BS 72, PP 100) by the combined cpDNA and *PgiC* data with two early diverging lineages, one consisting only of *Trachycalymma pseudofimbriatum*

and the other (Fig. 5; clade C5) consisting of the rest of the African *Asclepias* sensu lato (DI 2, BS 82, PP 100). Since *Trachycalymma pseudofimbriatum* is endemic to the highlands of southern Ethiopia and restricted only to montane grasslands at about 2700 m (Goyder 2001b), it must have split from the main lineage at the formation of suitable elevation and habitat conditions on the Ethiopian highlands. The Ethiopian highlands are believed to have begun to rise 75 million years ago (Kingdom 1989) but to have been sufficiently elevated and formed open grasslands and woodlands after the retreat of forests during the Miocene (23.03 to 5.33) as a result of rifting and uplift of the plateaus that border the rift zone (Axelrod and Raven 1978).

Within the large African clade (C5; Figs. 5 and 7), there are two lineages that are now more confidently resolved than with cpDNA alone (Chapter 1). The clade of the *Gomphocarpus integer* group plus *G. filiformis* (clade C18: Fig. 5,; DI 1, BS 82, PP 100 and Fig. 7, BS 85) is the next diverging lineage from the rest of the African *Asclepias* generic complex (Clade C6: Fig. 5, DI 1, BS 57, PP 99 and Fig. 7, BS 60). Within the smaller clade (C18, Figs. 5 and 7), which contains only narrow-leaved species, *G. filiformis* is the first diverging lineage, representing a colonization of the extremely arid sandy habitat of the Namib Desert and the Karoo (Goyder and Nicholas 2001). The rest of this clade further diverged into a northern lineage, including *G. stenophyllus* and *G. integer* in the semiarid, open, rocky ground and disturbed areas from Tanzania north to southern Ethiopia, and a southern lineage of *G. tenuifolius*, restricted to granite kopjes in Zimbabwe. This resolution within clade C18 supports the assertion (chapter 1) that clade C18 may have either been wide spread or

originated in southern Africa and then spread north. This hypothesis however still needs to be tested by a biogeographic analysis.

Further resolution that has been supported (Fig 5; DI 2, BS 82, PP 100 and Fig; 7 BS 90) by the addition of nuclear data is the placement of the *Gomphocarpus glaucophyllus* clade (C23) and a *Pachycarpus lineolatus-P. bisacculatus* clade (C24) as sister clades within clade C21. Clade C23 (Figs. 5 and 7), consists of *G. glaucophyllus* and *G. swynnertonii* which are sister to *G. praticolus*. All species in clade C21 are geographically widespread and broad-leaved. However the *Pachycarpus* species have petiolate leaves whereas the *Gomphocarpus* species have sessile or sub-sessile leaves. The *Gomphocarpus Pachycarpus* clade (C21, Figs. 5 and 7), has been resolved as sister to the *Glossostelma spathulatum*, which is also a broad leaved species. This relationship is slightly more strongly supported by the MP analysis when species showing conflicting placements between separate chloroplast and nuclear analyses were excluded (Fig. 7).

The genus *Miraglossum*, unsampled in our previous study, turns out to be closely related to *Aspidoglossum* since the two *Miraglossum* species form a clade C12 with species of *Aspidoglossum*, although with only weak support (C12: Fig. 5, BS 59, PP 100 and Fig. 7 BS 64) for this group. Taking into account the earlier placement (Chapter 1) of all species of *Aspidoglossum* in one clade with *Schizoglossum* species, which are not sampled here, it appears that at least some of the species of *Miraglossum* and *Aspidoglossum* (clade C12) with the addition of at least some *Schizoglossum* species (Chapter 1) may form one coherent group. These three genera have always been considered as closely related (Kupicha 1984). They all have similar

corona morphology characterised by possession of apical appendages of various shapes and orientations {Kupicha, 1984 #746}.

Asclepias randii Asclepias cucullata and *Asclepias aurea* which have also been considered as closely related based on corona morphology were resolved in different clades (Fig. 5, clades C9, C11 and C14; Fig. 7 by the combined analysis though with weak support. When *Asclepias cucullata* and *Asclepias aurea*, which were strongly supported as sister by the chlorplast phylogeny (chapter 1), were excluded from the analysis (Fig. 7), *Asclepias randii* was placed in a clade with *Stenostelma capense* and *Asclepias gibba* but without bootstrap support. More data are therefore required to confidently resolve the relationship of these species. However, the ML analysis with discordantly placed taxa excluded (Fig 8), resolved a better supported (ML BS 77) clade of the *Margaretta rosea-Stathmostelma pedunculatum* clade as sister to the *Aspidoglossum- Miraglossum* clade (C12). The remaining relationships of MP and ML trees (Figs. 5, 6 and 7) were either weakly or not supported and did not contradict the results of the chloroplast analysis (Chapter 1).

Phylogenetic value of the PgiC region

The level of sequence variation in the low-copy nuclear *PgiC* gene is higher than that of the cpDNA regions used previously in trying to resolve relationships within the Asclepiadinae. This is shown by the low relative number of informative characters (3.4%) for all the three plastid markers (*rpl16* intron, *rpoB-trnC*^{GCA} spacer, and *trnS*^{GCU}-*trnG*^{UUC} spacer/*trnG* intron) combined, whereas the partial *PgiC* (730-825 bp

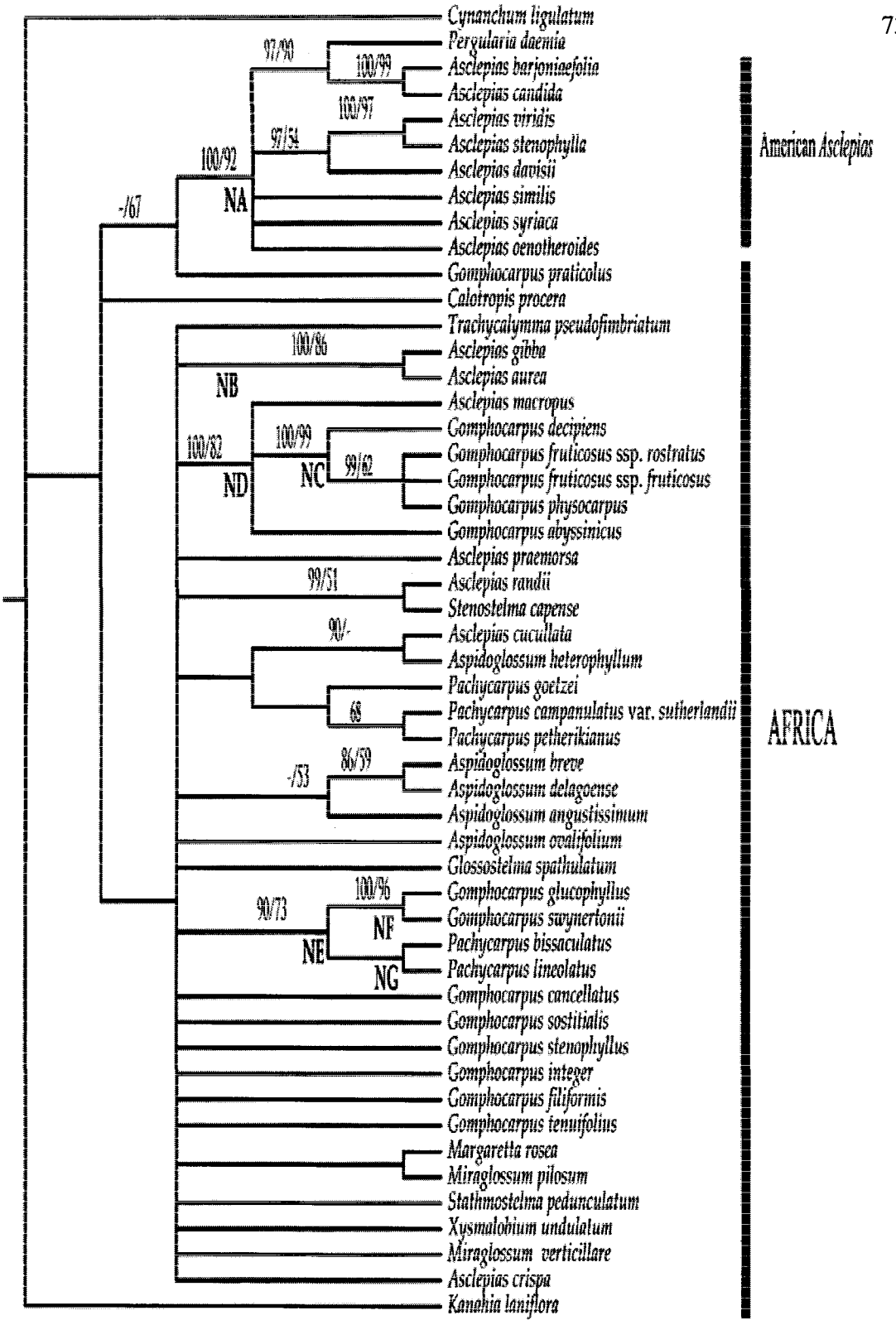
from between exons 11 and 16) yielded 9.6% in this study, even though the taxonomic range of our sampling was narrower here. There were also more parsimony informative indels (eight) in the *PgiC* region as compared to an average of three indels for each of the chloroplast regions. However, the low level of resolution and support in many parts of the *PgiC* tree can be explained by the relatively low consistency and retention indices (CI excluding uninformative characters = 0.5815, RI = 0.7551, RC= 0.5855), indicating a high number of homoplasious characters. Nevertheless, the *PgiC* gene region also yielded a number of autapomorphies that may be informative with increased taxonomic sampling.

Concluding remarks

Resolution of the African Asclepiadinae phylogeny by cpDNA has been challenging. Our study using partial *PgiC* sequences has recovered some novel relationships for the African *Asclepias* s.l. and has teased out early diverging lineage within the African *Asclepias* s.l.

It also seems plausible that the short internal and terminal branches in the African *Asclepias* complex (Fig. 4) may actually be a reflection of a recent rapid radiation in which species have originated almost simultaneously over a short period resulting in the low DNA sequence divergence exhibited by our data. However, partial sequences of the low-copy nuclear *PgiC* gene that we added to the cpDNA have increased support for both basal nodes (Fig 5. nodes C1 and C5) and internal nodes (Figs. 5 and 7 e.g. C18, C19, C20, and C22). Addition of *PgiC* sequences has also resolved some

previously unresolved basal relationships (Fig 5. node C4 and C21). The corroboration of the cpDNA phylogenetic hypotheses by the *PgiC* has made it possible to more confidently infer a number of evolutionary phenomena from the estimated topology. Finally, this study also demonstrates the usefulness of nuclear *PgiC* sequences for resolving some of the relationships that are difficult to resolve by multiple cpDNA regions only and therefore suggests that addition of more nuclear regions promises to achieve almost complete resolution of the African Asclepiadinae phylogeny.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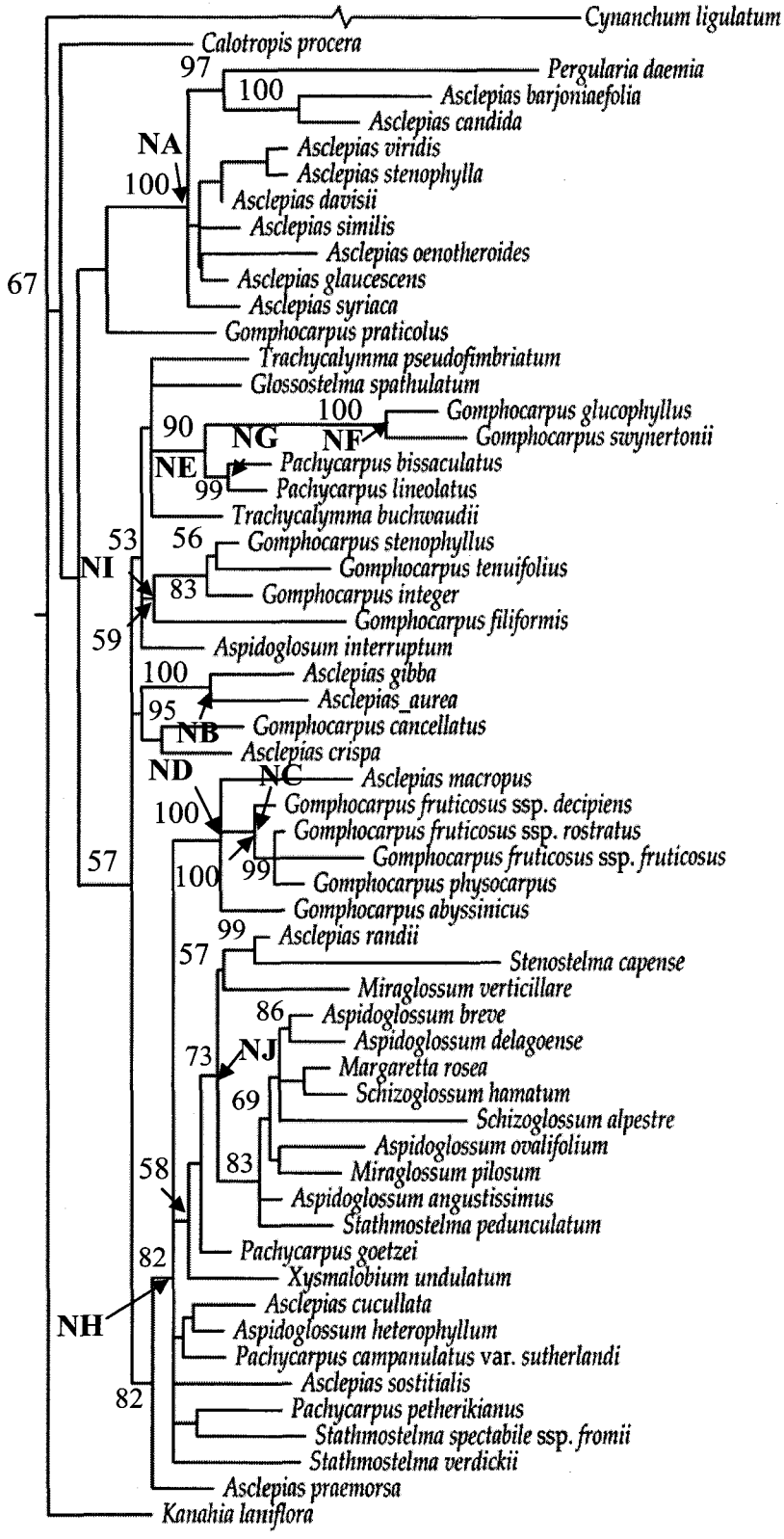
Asclepias syriaca
Asclepias oenotheroides
Gomphocarpus praticolus
Calotropis procera
Trachycalymma pseudofimbriatum
Asclepias gibba
Asclepias aurea
Asclepias macropus
Gomphocarpus decipiens
Gomphocarpus fruticosus ssp. rostratus
Gomphocarpus fruticosus ssp. fruticosus
Gomphocarpus physocarpus
Gomphocarpus abyssinicus
Asclepias praemorsa
Asclepias randii
Stenostelma capense
Asclepias cucullata
Aspidoglossum heterophyllum
Pachycarpus goetzei
Pachycarpus campanulatus var. sutherlandii
Pachycarpus petherikianus
Aspidoglossum breve
Aspidoglossum delagoense
Aspidoglossum angustissimum
Aspidoglossum ovalifolium
Glossostelma spathulatum
Gomphocarpus glucophyllus
Gomphocarpus swynertonii
Pachycarpus bissaculatus
Pachycarpus lineolatus
Gomphocarpus cancellatus
Gomphocarpus sostitialis
Gomphocarpus stenophyllus
Gomphocarpus integer
Gomphocarpus filiformis
Gomphocarpus tenuifolius
Margaretta rosea
Miraglossum pilosum
Stathmostelma pedunculatum
Xysmalobium undulatum
Miraglossum verticillare
Asclepias crispa
Kanahia laniflora
American Asclepias
AFRICA
NA
NB
NC
ND
NE
NF
NG
97/90
100/99
100/97
97/54
100/92
-/67
100/86
100/82
100/99
99/62
99/51
90/-
68
86/59
-/53
100/%
90/73

Fig. 3

FIG. 3: Maximum parsimony strict consensus tree of the African *Asclepias* generic complex based on partial sequences of the low copy nuclear *PgiC* gene. Numbers separated by a forward slash are Bayesian posterior probabilities on the left and bootstrap values on the right. Letters represent the corresponding nodes and their respective clades.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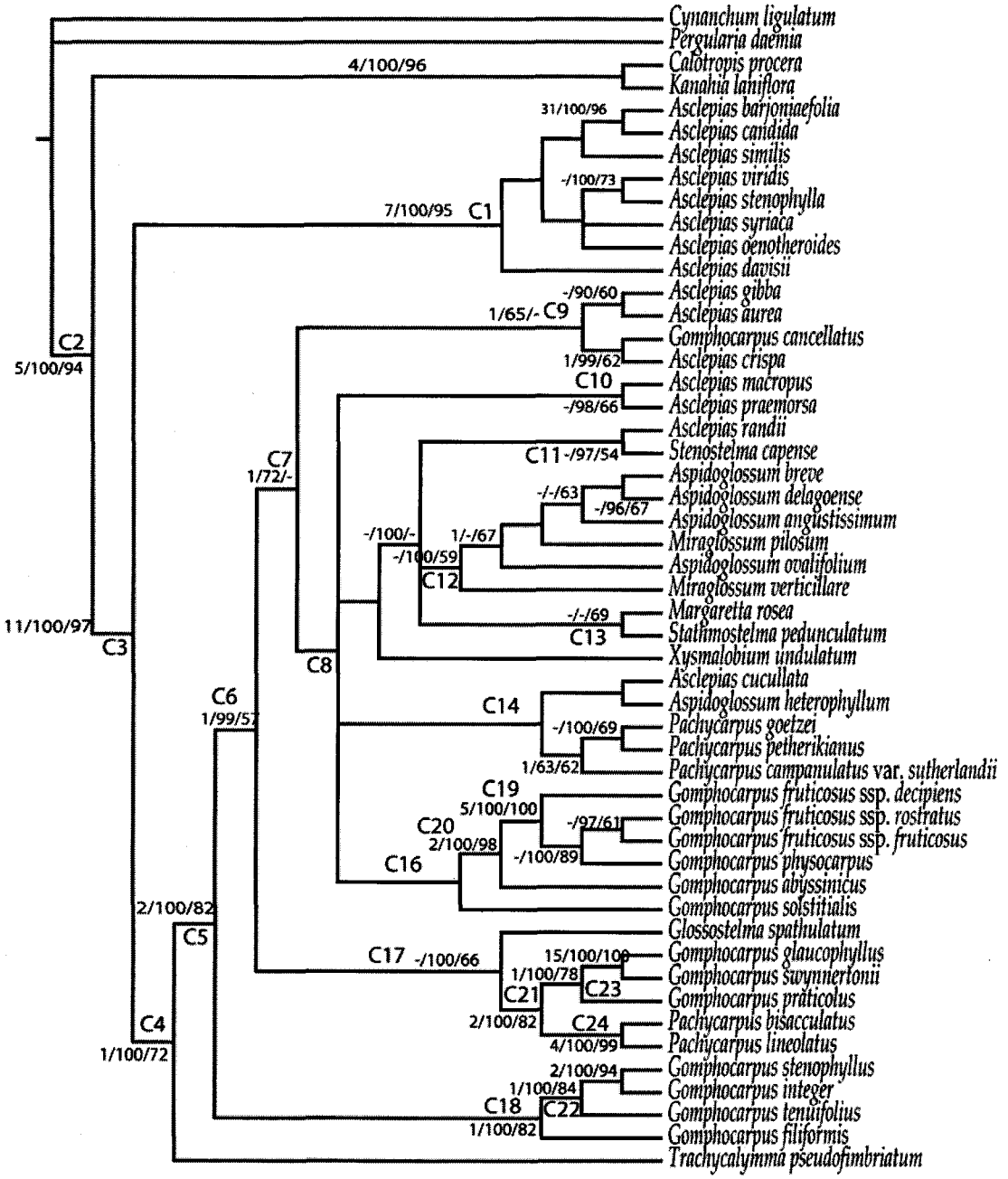
Asclepias similis
Asclepias oenotheroides
Asclepias glaucescens
Asclepias syriaca
Gomphocarpus praticolus
Trachycalymma pseudofimbriatum
Glossostelma spathulatum
Gomphocarpus glucophyllus
Gomphocarpus swynertonii
Pachycarpus bissaculatus
Pachycarpus lineolatus
Trachycalymma buchwaudii
Gomphocarpus stenophyllus
Gomphocarpus tenuifolius
Gomphocarpus integer
Gomphocarpus filiformis
Aspidoglosum interruptum
Asclepias gibba
Asclepias_aurea
Gomphocarpus cancellatus
Asclepias crispa
Asclepias macropus
Gomphocarpus fruticosus ssp. decipiens
Gomphocarpus fruticosus ssp. rostratus
Gomphocarpus fruticosus ssp. fruticosus
Gomphocarpus physocarpus
Gomphocarpus abyssinicus
Asclepias randii
Stenostelma capense
Miraglossum verticillare
Aspidoglossum breve
Aspidoglossum delagoense
Margaretta rosea
Schizoglossum hamatum
Schizoglossum alpestre
Aspidoglossum ovalifolium
Miraglossum pilosum
Aspidoglossum angustissimus
Stathmostelma pedunculatum
Pachycarpus goetzei
Xysmalobium undulatum
Asclepias cucullata
Aspidoglossum heterophyllum
Pachycarpus campanulatus var. sutherlandi
Asclepias sostitialis
Pachycarpus petherikianus
Stathmostelma spectabile ssp. fromii
Stathmostelma verdickii
Asclepias praemorsa
Kanahia laniflora
NA
NB
NC
ND
NE
NF
NG
NH
NI
NJ
0.02 substitutions/site

Fig. 4

FIG. 4: Maximum likelihood phylogram of the African *Asclepias* generic complex inferred by ML ratchet method from partial sequences of the low copy nuclear *PgiC* gene. Numbers on the nodes are Bayesian poeterior probabilities.


Cynanchum ligulatum
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Asclepias gibba
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Gomphocarpus cancellatus
Asclepias crispa
Asclepias macropus
Asclepias praemorsa
Asclepias randii
Stenostelma capense
Aspidoglossum breve
Aspidoglossum delagoense
Aspidoglossum angustissimum
Miraglossum pilosum
Aspidoglossum ovalifolium
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Pachycarpus goetzei
Pachycarpus petherikianus
Pachycarpus campanulatus var. sutherlandii
Gomphocarpus fruticosus ssp. decipiens
Gomphocarpus fruticosus ssp. rostratus
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Gomphocarpus abyssinicus
Gomphocarpus solstitialis
Glossostelma spathulatum
Gomphocarpus glaucophyllus
Gomphocarpus swynnertonii
Gomphocarpus praticolus
Pachycarpus bisacculatus
Pachycarpus lineolatus
Gomphocarpus stenophyllus
Gomphocarpus integer
Gomphocarpus tenuifolius
Gomphocarpus filiformis
Trachycalymma pseudofimbriatum
4/100/96
31/100/96
-/100/73
7/100/95
C1
-/90/60
1/65/-
C9
1/99/62
C2
5/100/94
C10
-/98/66
C11
-/97/54
C7
1/72/-
-/-/63
-/96/67
-/100/-
1/-/67
-/100/59
C12
-/-/69
C13
11/100/97
C3
C8
C6
1/99/57
C14
-/100/69
1/63/62
C19
5/100/100
-/97/61
C20
2/100/98
-/100/89
C16
2/100/82
C5
C17
-/100/66
15/100/100
1/100/78
C21
C23
2/100/82
C24
4/100/99
C4
1/100/72
2/100/94
1/100/84
C18
C22
1/100/82

Fig. 5

FIG. 5: Maximum parsimony strict consensus tree of the African *Asclepias* generic complex based on combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene. Numbers separated by a forward slash (from left to right) represent Decay index, Bayesian posterior probabilities and bootstrap values respectively. Letters represent the corresponding nodes and their respective clades.

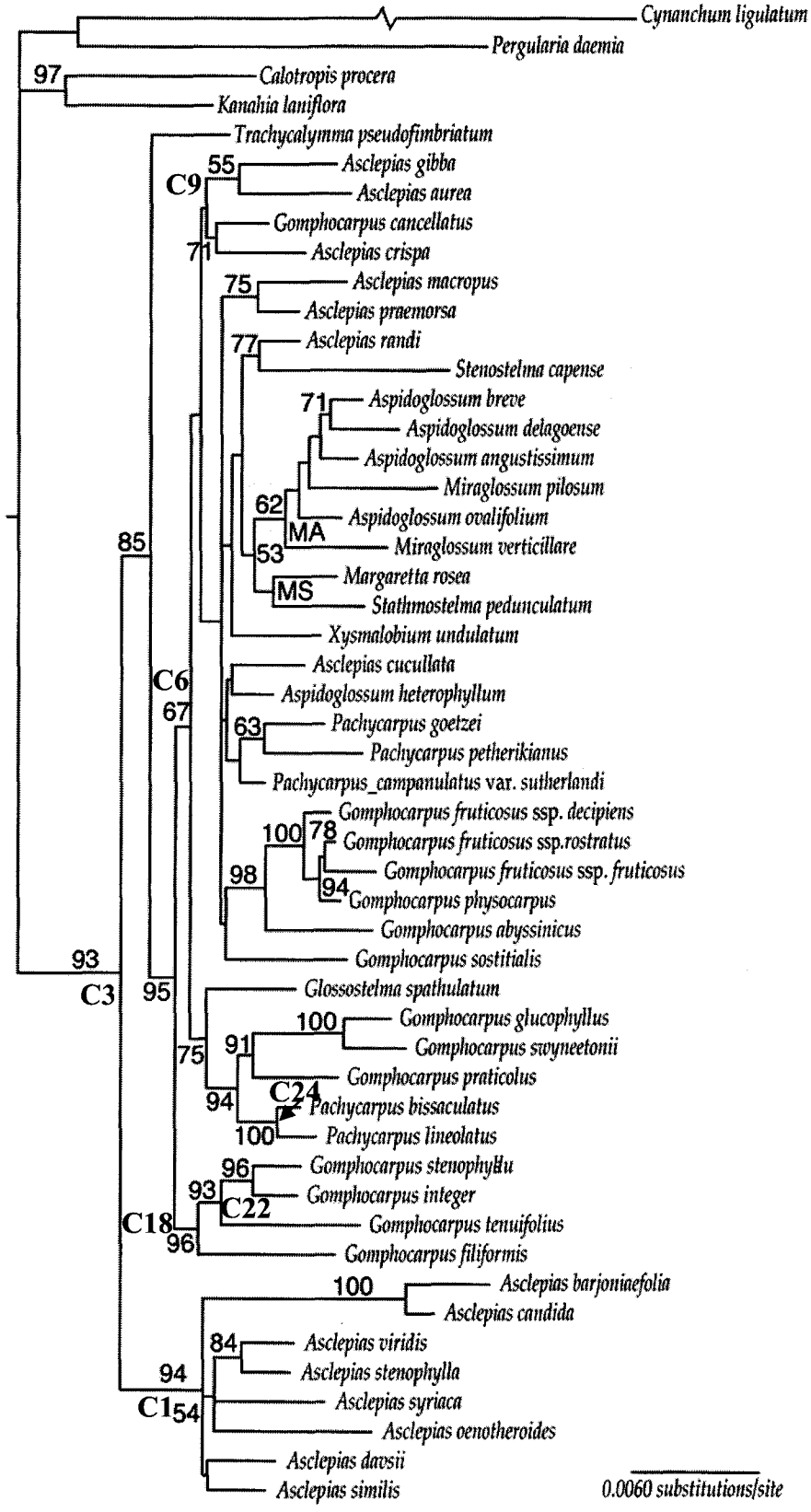
Cynanchum ligulatum
Pergularia daemia
97
Calotropis procera
Kanahia laniflora
Trachycalymma pseudofimbriatum
55
C9
Asclepias gibba
Asclepias aurea
Gomphocarpus cancellatus
71
Asclepias crispa
75
Asclepias macropus
Asclepias praemorsa
77
Asclepias randi
Stenostelma capense
71
Aspidoglossum breve
Aspidoglossum delagoense
Aspidoglossum angustissimum
Miraglossum pilosum
62
MA
Aspidoglossum ovalifolium
85
53
Miraglossum verticillare
MS
Margaretta rosea
Stathmostelma pedunculatum
Xysmalobium undulatum
Asclepias cucullata
C6
Aspidoglossum heterophyllum
67
63
Pachycarpus goetzei
Pachycarpus petherikianus
Pachycarpus_campanulatus var. sutherlandi
Gomphocarpus fruticosus ssp. decipiens
100
78
Gomphocarpus fruticosus ssp.rostratus
98
Gomphocarpus fruticosus ssp. fruticosus
94
Gomphocarpus physocarpus
Gomphocarpus abyssinicus
93
Gomphocarpus sostitialis
C3
95
Glossostelma spathulatum
100
Gomphocarpus glucophyllus
91
Gomphocarpus swyneetonii
75
Gomphocarpus praticolus
C24
94
Pachycarpus bissaculatus
100
Pachycarpus lineolatus
96
Gomphocarpus stenophyllu
93
Gomphocarpus integer
C22
C18
Gomphocarpus tenuifolius
96
Gomphocarpus filiformis
100
Asclepias barjoniaefolia
Asclepias candida
84
Asclepias viridis
94
Asclepias stenophylla
C154
Asclepias syriaca
Asclepias oenotheroides
Asclepias davsii
Asclepias similis
0.0060 substitutions/site

Fig. 6

FIG. 6: Maximum likelihood phylogram of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G*/*trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene. Letters represent the nodes and their respective clades. Numbers on the nodes are ML bootstrap values estimated with RaXML.

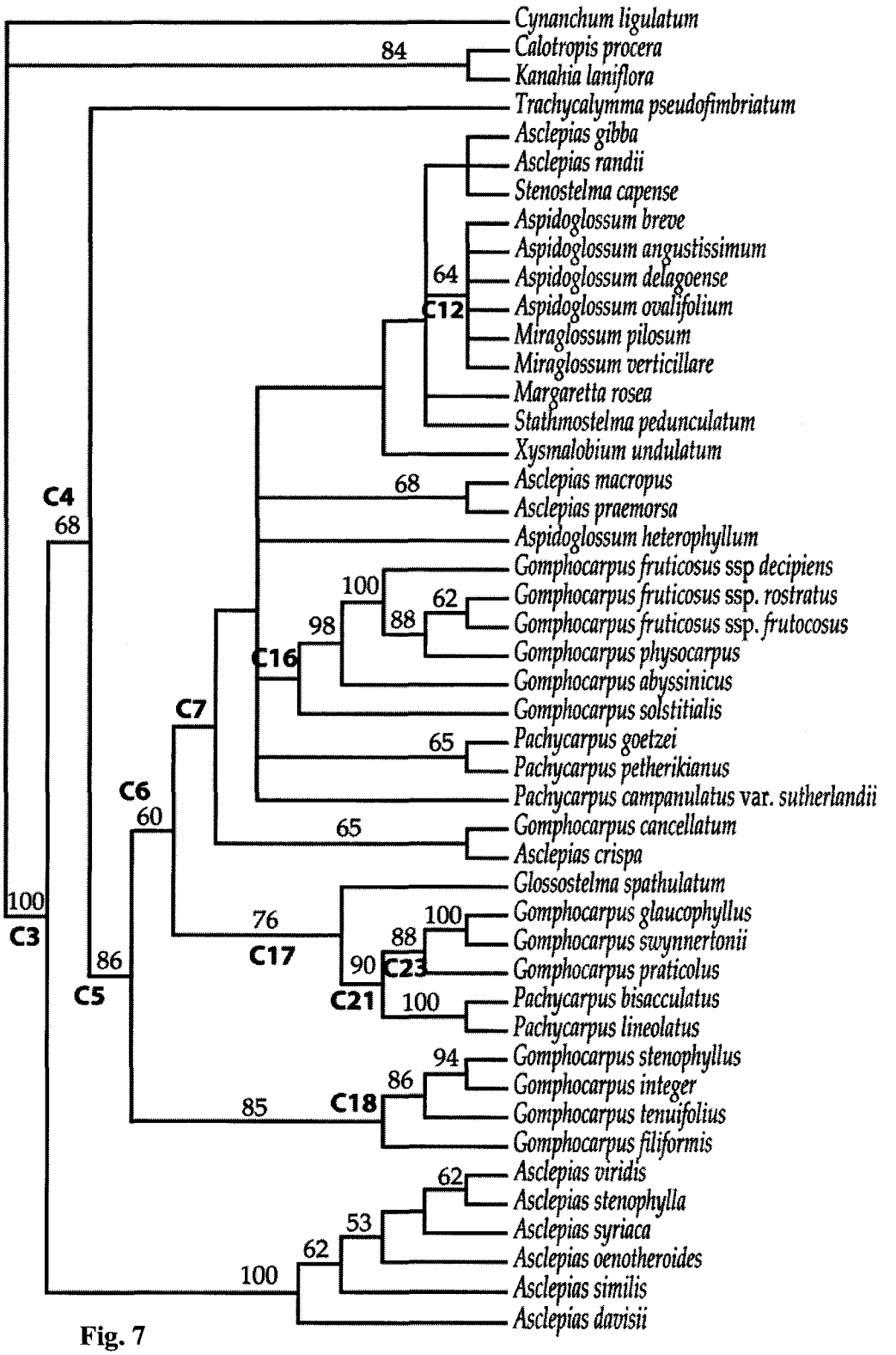
Cynanchum ligulatum
Calotropis procera
Kanahia laniflora
Trachycalymma pseudofimbriatum
Asclepias gibba
Asclepias randii
Stenostelma capense
Aspidoglossum breve
Aspidoglossum angustissimum
Aspidoglossum delagoense
Aspidoglossum ovalifolium
Miraglossum pilosum
Miraglossum verticillare
Margaretta rosea
Stathmostelma pedunculatum
Xysmalobium undulatum
Asclepias macropus
Asclepias praemorsa
Aspidoglossum heterophyllum
Gomphocarpus fruticosus ssp decipiens
Gomphocarpus fruticosus ssp. rostratus
Gomphocarpus fruticosus ssp. frutocosus
Gomphocarpus physocarpus
Gomphocarpus abyssinicus
Gomphocarpus solstitialis
Pachycarpus goetzei
Pachycarpus petherikianus
Pachycarpus campanulatus var. sutherlandii
Gomphocarpus cancellatum
Asclepias crispa
Glossostelma spathulatum
Gomphocarpus glaucophyllus
Gomphocarpus swynnertonii
Gomphocarpus praticolus
Pachycarpus bisaculatus
Pachycarpus lineolatus
Gomphocarpus stenophyllus
Gomphocarpus integer
Gomphocarpus tenuifolius
Gomphocarpus filiformis
Asclepias viridis
Asclepias stenophylla
Asclepias syriaca
Asclepias oenotheroides
Asclepias similis
Asclepias davisii
84
64
C12
68
C4
68
100
62
88
98
C16
C7
65
C6
60
65
100
C3
76
C17
100
88
86
90
C23
C5
C21
100
94
86
85
C18
62
53
62
100

Fig. 7

FIG. 7 Maximum parsimony strict consensus tree of the African *Asclepias* generic complex based on combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene but with taxa that had incongruent placement between the nuclear and Chloroplast analyses excluded. Numbers represent MP bootstrap values.

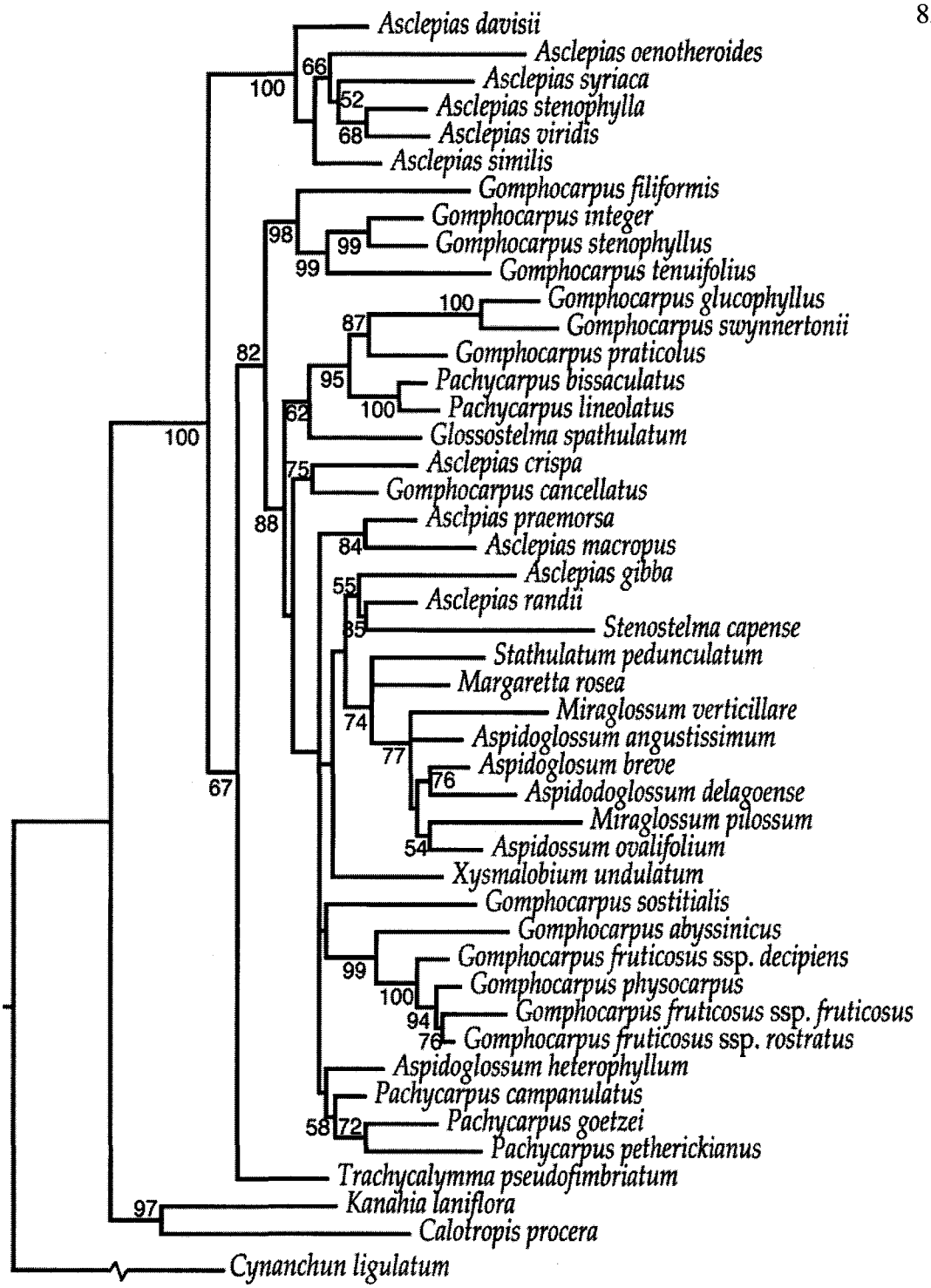
Asclepias davisii
Asclepias oenotheroides
Asclepias syriaca
Asclepias stenophylla
Asclepias viridis
Asclepias similis
Gomphocarpus filiformis
Gomphocarpus integer
Gomphocarpus stenophyllus
Gomphocarpus tenuifolius
Gomphocarpus glucophyllus
Gomphocarpus swynnertonii
Gomphocarpus praticolus
Pachycarpus bissaculatus
Pachycarpus lineolatus
Glossostelma spathulatum
Asclepias crispa
Gomphocarpus cancellatus
Asclpias praemorsa
Asclepias macropus
Asclepias gibba
Asclepias randii
Stenostelma capense
Stathulatum pedunculatum
Margaretta rosea
Miraglossum verticillare
Aspidoglossum angustissimum
Aspidoglosum breve
Aspidodoglossum delagoense
Miraglossum pilossum
Aspidossum ovalifolium
Xysmalobium undulatum
Gomphocarpus sostitialis
Gomphocarpus abyssinicus
Gomphocarpus fruticosus ssp. decipiens
Gomphocarpus physocarpus
Gomphocarpus fruticosus ssp. fruticosus
Gomphocarpus fruticosus ssp. rostratus
Aspidoglossum heterophyllum
Pachycarpus campanulatus
Pachycarpus goetzei
Pachycarpus petherickianus
Trachycalymma pseudofimbriatum
Kanahia laniflora
Calotropis procera
Cynanchun ligulatum
0.0060 substitutions per site

Fig. 8

FIG. 8 Maximum Likelihood tree of the African *Asclepias* generic complex based on combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene but with taxa that had incongruent placement between the nuclear and Chloroplast analyses excluded. Numbers represent ML bootstrap values.

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TABLE 5: Primers utilized for DNA amplification and sequencing of the *PgiC* region.

Primer Name	**Primer sequence**	**Putative utility**	**Source**
AA16R	CCY TTN CCR TTR CTY TCC AT	Universal	(Ford et al. 2006)
Asc11F	CTG GGT AGG AGG ACG ATA TAG	Asclepiadinae	this study
Asc16R	CCA TGC TAA CCT GGA TAA GCC	Asclepiadinae	this study
Asc14R	GCT CAA CAA TCC CAG GAG AAC	Asclepiadinae	this study
Asc13F	GCA TTT CAG CTC AGC ACC ATT CG	Asclepiadinae	this study

TABLE 6. Summary statistics of the aligned datasets of the partial sequences of the nuclear *PgiC* gene and three chloroplast regions (*trnS-trnG* intergenic spacer/*trnG* intron, *trnC-rpoB* intergenic spacer, and *rpl16* intron) for 51 taxa of the Asclepiadinae.

	***cpDNA (trnS-trnG /trnG, trnC-rpoB, rpl16* intron) sequences**	***PgiC sequences***	**Combined Sequences**
Total length of aligned sequences	4212	1372	5584
Number of indels coded	9	8	17
Parsimony informative characters	144 (3.4%)	132 (9.6%)	259 (4.6%)
Number of ambiguously aligned sequence positions that were omitted from analysis	218	41	259

CHAPTER 3: EVOLUTION OF MORPHOLOGY AND HABITAT PREFERENCE IN THE AFRICAN ASCLEPIAS COMPLEX (APOCYNACEAE)

EVOLUTION OF MORPHOLOGICAL AND HABITAT PREERENCE IN THE AFRICAN ASCLEPIAS COMPLEX (APOCYNACEAE)

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ABSTRACT

Genera delimitation in the African *Asclepias* generic complex has been based on morphological characters that are usually not exclusive to any particular clade or genus. The evolution of these characters has not been assessed in a phylogenetic context using an independent set of characters such as molecular characters. Recent phylogenetic analyses of the group have provided a framework for determining the evolution of the morphological characters. This study employs the maximum parsimony algorithm to reconstruct character evolution of some morphological characters that have been utilized for generic delimitations in the African *Asclepias* complex. Evolution of species habitat preference is also reconstructed. Ancestral character reconstruction was done by optimizing six morphological characters and one habitat character obtained from literature, herbarium specimens and photographs, onto a phylogenetic tree of 51 species of the African (42 species) and American (six species) Asclepiadinae, inferred from sequences of three chloroplast and one nuclear DNA region. The morphological characters optimized on the phylogenetic tree were all homoplasious, as indicated by multiple origins. The surprising parallel in many characters among some species suggests a high propensity for convergent evolution in morphological characters in the African Asclepiadinae. Ancestral characters states included stems branching, broad leaves, presence of nodal only inflorescences, pedunculate inflorescences, reflexed petals and *Gomphocarpus* type cucullate plus laterally flattened corona lobes. Derived states included loss of branching, narrow leaves, presence of both nodal and terminal, inflorescences, terminal only

inflorescences, sessile inflorescence, non-reflexed petals and dorsiventrally flattened corona lobes and other cucullate corona forms.

INTRODUCTION

In plant groups where stable taxonomic classifications based on morphology have tended to elude taxonomists, high levels of homoplasy have commonly been found to be responsible for the difficulty in defining taxa e.g. (Garcia-Jacas et al. 2001; Kiel et al. 2006,Baker et al. 2000 Moylan et al. 2004). This is the case for the African *Asclepias* complex, a plant group that has a wide distribution in Africa and South west Asia. The African *Asclepias* complex have two main centers of diversity, one in East Africa and another in southern Africa (Liede 1997). The current concepts of African *Asclepias* complex genera are based on thorough but intuitive assessments of morphological characters, which have been found have been found not to be exclusive to any particular clade. Unique morphological synapomorphies have been hard to find among genera of the African *Asclepias* s.l. leading to utilization of suites of characters for delimiting genera (e.g. Goyder 1998a; Goyder 1998b; Goyder 2001b; Goyder 2005; Kupicha 1984; Nicholas and Goyder 1992).

Within the African *Asclepias* complex different characters have been used at different levels and in different groups. For example, Goyder (1994) has utilized corona morphology only for species delimitations within *Glossostelma* whereas for delimitation of the genus, he employed a different suite of characters in which none were unique to the genus. These characters included erect stems coming up after fires from narrow tuberous perennial rootstocks, with fusiform lateral leaves almost or

entirely glabrous; umbelliform inflorescence with campanulate corollas; gynostegia that are generally stipulate with a stout stalk; pollinaria with ovoid corpuscular, geniculate and flattened translator arms and smooth lanceolate follicles frequently attenuate at both ends (Goyder, 1994). Other genera were circumscribed based on other characters. For example, *Stathmostelma* (fig. 9A) is characterized with pollinaria possessing broadly winged and contorted translator arms and the anther wings that have convex margins and basal tails (Goyder 1998b) whereas *Pachycarpus* (fig. 9B) is characterized by stout stems, tuberous rootstocks, leaves with an indumentum of stiff hairs, dorsally flattened corona lobes with a variety of fleshy keels on the upper surface and follicles with longitudinal wings (Goyder 1998a). *Trachycalymma* is characterized by nodding or rarely subglobose inflorescences, corona lobes that arise at the base of the column and are adnate to it to the base of the anther wings and the vegetative habit of the plant with few annual stems arising from a tuberous rootstock (Goyder 2001b). Brown (1811) separated *Gomphocarpus* from Asclepias and sank most of the species of the genus *Gomphocarpus* into *Asclepias* based on *Asclepias* being composed of species with a horn or other appendage within the cucullate or folded part of the corona lobes and *Gomphocarpus* being species without a horn as the only distinguishing feature. *Gomphocarpus* is currently delimited by a suite of characters. These include; plants with a non tuberous rootstock, nodding extra axillary inflorescences and cucullate corona lobes that generally lack a tooth (Goyder and Nicholas 2001). *Stenostelma* species are defined by having linear or filiform leaves that are slightly ascending, a xerophytic appearance and a gynostegium concealed in corolla and exhibiting a simple corona which is

rounded at the apex. *Miraglossum is* distinguished by its slender habit with linear leaves that are almost appressed and parallel to the stem, flattened ribbon-like translator arms and a corona that is erect and possesses apical processes that are sometimes quite twisted and sometimes branched, as in *M. superbum* (Kupicha 1984). *Aspidoglossum* (Fig. 9 E, F) has been defined by sessile fasciculate inflorescences and suasage shaped polinia that are subapically attached to translator arms (Kupicha 1984).

However, the evolution of these characters used to define genera has not been evaluated phylogenetically using an independent set of characters such as molecular characters. The estimation of phylogeny for the African *Asclepias* generic complex (Goyder et al. 2007), Chapters 1 and 2) has provided a framework for understanding the evolution of floral and vegetative characters utilized for generic delimitations in the African *Asclepias* s.l. and those that may newly have potential taxonomic utility for the group.

Species of the African *Asclepias* complex occur in a number of habitats ranging from different variants of grasslands to variants of open woodlands (Goyder 1998a; Goyder 1998b; Goyder 2001; Goyder and Nicholas 2001; Kupicha 1984; Ollerton et al. 2003; Smith 1988). These includes seasonally waterlogged grasslands, regularly burned highland or montane grasslands, highveild grasslands, mistbelt rocky grassland, *Themeda* coastal grassland, grassy slopes, scrub or riparian woodland, open *Protea* scrub, *Brachystegia* woodlands, open *Brachystegia/Uapaca* woodland and other types of mixed open deciduous woodland. Other habitats include open rocky ground, stoney hillsides, dry fallow land and disturbed areas such as alongside highways, bridges and

railway lines. Some species have been reported in extremely arid environments such as the Namib desert and the Karoo, but apparently following dry water courses in some areas (Goyder and Nicholas 2001). This study focuses only on one aspect of all these various habits namely whether the substrate in which plants grow is associated with sand, loam or black peaty soils, rocky or limestone ground or various combinations of these. Species have been reported as occurring in such specific substrates as black peaty soils, well drained soils, sandy soils, sand-loam soils, granite kopjes, rocky Hillsides, rock crevices, river banks and on dry shores of lakes or rivers. Sandy soils or rocky soils tend to be well drained soils whereas non sandy or non rocky soils tend to be less well drained. This study therefore investigates species evolution in relation to well drained (sandy or rocky) or poorly drained (non sandy, non rocky) substrates.

The purpose of this study is to (1) reconstruct morphological character evolution and (2) reconstruct evolution of African Asclepias species in relation to habitats with well drained and poorly drained substrates as inferred from character optimizations on the newly inferred molecular phylogeny (chapters 1 and 2) for the African *Asclepias* complex.

MATERIALS AND METHODS

Character coding and optimization

Six morphological characters and one habitat character were scored for 39 ingroup species within the African Asclepiadinae, three African Asclepiadinae outgroups,

eight species of America *Asclepias* and *Cynanchum ligulatum* (Table 7). The characters were mostly obtained from literature descriptions (Burrows and Willis 2005; Goyder 1998b; Goyder 2001b; Goyder and Nicholas 2001; Kupicha 1984; Langley 1980). The information from the literature was supplemented with personal observations from herbarium specimens and collector's notes. These specimens were obtained from Makerere University Herbarium (MHU), National Botanical Research Institute National Herbarium of Namibia (WIND) and University of Zambia Herbarium (UZL). Photographs from the Royal Botanic Gardens, Kew Herbarium online Catalogue (http://apps.kew.org/herbcat/navigator.do), pictures taken in the field and pictures from the internet were used to supplement the information from herbarium sources. Photographs from the internet were only used in cases where confident identification could be confirmed. Details of the sources of information are given in appendix B. Descriptions of character coding are given below. Some characters coded tended to vary in a continuous manner in single individuals for example Leaf size in *Aspidoglossum heterophyllum* varies from narrowly linear to broadly elliptic and therefore possess both narrow and broad leaves as defined here. Such continuously varying characters were coded as having multiple states (more than one character condition/state occuring in one species). It was essential to explore both vegetative and flora characters because floral characters have been used in taxonomic circumscriptions of these taxa and vegetative characters were found by Goyder et al (2007) to be a more reliable guide to evolutionary relationships than the traditional floral characters. The seven coded characters (Table 7) were traced using simple parsimony onto the maximum likelihood phylogenetic tree obtained from the analysis

of combined chloroplast and nuclear DNA sequences (Chapter 2) using MacClade 4.08 (Maddison and Maddison 2005). To further determine how reconstruction of ancestral states might have been affected by phylogenetic uncertainty, The seven characters were each mapped on 100 randomly selected trees obtained by Bayesian phylogenetic analysis. These were each examined to check if they supported the various hypotheses of character evolution. Proportions of trees showing the ancestral character reconstructions hypothesized by reconstruction on the ML tree were taken as support for the respective hypotheses.

1. Stem branching— stems always branched (0); stems never branched (1); stems sometimes branched (2)

Species of the group may be branched as in most species of *Gomphocarpus* (Goyder and Nicholas 2001) or not branched with a solitary stem as in *Xysmalobium undulatum* (Langley 1980), but sometimes with several annual stems arising from a slender or stout horizontal or vertical underground root structure as in *Stathmostelma spectabile* (Goyder 1998b). There are several aspects of stem branching observed in the African Asclepiadinae. Some species are only occasionally branched e.g. *Schizoglossum hamatum* (Kupicha 1984), *Stathmostelma pedunculatum* (Goyder 1998b) and *Margaretta rosea* (Goyder 2005) whereas others such as *Stathmostelma fornicatum* (Goyder 1998b) are generally branched but occasionally unbranched. Many branched species exhibit basal branching as in for example some species of *Stathmostelma* (Goyder 1998b) or *Xysmalobium* (Langley 1980) and *Trachycalymma* (Goyder

2001b). Other species generally show apical branching e.g. *Gomphocarpus physocarpus* (Goyder and Nicholas 2001). It is not clear how the condition of being unbranched and solitary relates to several unbranched stems arising from a single rootstock. Not enough information was available in the literature for number of stems arising from the rootstock of branched stems for such a character to be coded and therefore evolution of variation in number of stems arising from the rootstock has not been investigated here.

2. Leaf type— leaves broad (0); leaves narrow (1) multistate leaves (both narrow and broad leaves in a species) (2).

The African Asclepiadinae exhibit a variety of leaf shapes including narrowly linear, linear-lanceolate, lanceolate, elliptic, oblanceiolate, spathulate, oblong, oval, triangular, trullate and suborbicular or different variations of these shapes. However, the plants exhibit either broad leaves or narrow as described below. The leaf shapes described in the literature and /or observed on herbarium specimens and pictures to be lanceolate, elliptic, oblanceiolate, spathulate, oblong, oval, triangular, trullate, suborbicular and any broad variations thereof (approximate width to length ratio= 3 or less) were considered as broad leaves. Those described in the literature and /or observed on herbarium specimens and pictures as being linear and linear lanceolate and any narrow variations thereof were considered to be narrow leaves (approximate width to length ratio= 6 or greater). Some species such as *P. lineolatus* (possessing narrowlly lanceolate to broadly ovate, elliptic or oblong) and *Pachycarpus*

campanulatus (possessing narrowly linear to lanceolate leaves) are treated here as having multistate leaves (both narrow and broad leaves in a species).

Floral characters

3. Inflorescence position— nodal (extra-axillary) only (0); nodal (extra axillary) and terminal (1); terminal only (2)

4. Inflorescence attachment to stem— inflorescence sessile (0); inflorescence pedunculate (1)

Inflorescences in the Asclepiadinae are mostly nodal (pedunculate and extra axillary or sessile and fasciculate) and terminal but sometimes only terminal. There are many variations to the position and attachment of the inflorescences to the stem in different species. Most species possess only pedunculate extra axillary inflorescences and some have additional terminal inflorescences. Other species only possess terminal inflorescences. Other species have nodal sessile fasciculate inflorescences as in species of *Aspidoglossum* and *Miraglossum* (Kupicha 1984). Inflorescences may also be positioned in the upper third of the stem only or may extend further down the stem. There are other aspects of inflorescence that have not been considered here such as the orientation of the inflorescence (e.g. erect versus nodding), number of flowers per inflorescence, length of the peduncle and pedicels and overall shape of the inflorescence (e.g. globose, hemispherical or all pedicels parallel or sub parallel.

These characters could be investigated in future studies when obtaining such data becomes feasible.

5. Orientation of petals— reflexed but not rolled back on themselves longitudinally (0); generally campanulate with individual lobes often reflexed at the tips (1) reflexed and in rolled back on themselves longitudinally (2); rotate, saucer shaped, or spreading to slightly reflexed (3); erect at the base revolute in the distal half (4); globose or globose campanulate or campanulate and lobed in the upper half (5)

The corolla in Asclepiadinae exhibits diverse shapes and orientation that characterize different species. One corolla characteristic in the Asclepiadinae is tendency in some species for the corolla to be reflexed to various degrees. The various corolla orientations include rotate to slightly reflexed petals on the same plant (e.g. *Stathmostelma pedunculatum*), rotate or saucer shaped (e.g. *P. lineolatus P. bisacculatus*), spreading (e.g. *Aspidoglossum heterophyllum)*, erect at the base revolute in the distal half (e.g. *Margaretta rosea*), campanulate (e.g. *P goetzei, Pachycarpus petherickianus*), globose or globose campanulate (e.g. *P. campanulatus*) and campanulate with individual lobes often reflexed at the tips (e.g. *Glossostelma spathulatum*). There may be slight variations to each of these general types. For example in addition to being spreading or slightly reflexed, species of *Stathmostelma* possess corollas that are characteristically rolled back on themselves longitudinally (Goyder 1998b) giving them an easily distinguishable appearance from other similarly

oriented corolla types. In this study a corolla is taken to be reflexed if it is oriented away from the gynostegium by at least a 95 degree angle.

6. Shape of the corona lobe— with dorsiventrally flattened horizontal plate spreading out from gynostegium, sometimes with vertical plates arising from the upper surface or base at the proximal end (0) laterally flattened and overtopping the column (1) cucullate or at least grooved on apex with proximal margin divided into a pair of inward pointing teeth reaching over the gynostegium head; (2); not hooded or cucullate and with upper half arched over the head of the column (3); arising from base of column, with fimbriate or papillose margins (4); lobes dorsiventrally flattened not spreading away from column and with a single straight or curved apical processes sometimes overlapping neighboring processes (5); lobes dorsiventrally flattened not spreading away from column and with one or two apical processes (6); laterally flattened and equal or overtopping the column but with a basal spur (7). Corona lobes saccate or cucullate with a prominent free or adnate horn (8)

Corona morphology has so far played a major role in combination with other characters in the delimitation of genera in the African Asclepiadinae. Shapes of the corona vary greatly from relatively simple forms such as in *Glossostelma* (Goyder 1995) to highly elaborate ones such as in species of *Miraglossum* (Kupicha 1984). However, the various corona characters even though utilized for generic delimitations can not all be optimized on the phylogeny in this study due to challenges inherent in coding characters from literature and available herbarium specimens alone. Such challenges include determination of homology of some structures described in the

literature such as the variously positioned horns, and the impossibility of examining some characters in the absence of actual floral material. Therefore, characters that could not adequately be coded will await a careful and thorough examination on spirit and/or field material in future studies.

In some genera corona lobes are generally similar. For example *Stathmostelma* corona lobes are concave-cucullate with the inner apical margins usually producing a pair of inward pointing teeth. However in other genera such as *Glossostelma* coronas are variously shaped sometimes dorsally compressed or ventrally compressed (only one species was sampled) but always with the apical portion inflexed or arched over the column to as much as half its length (Goyder 1995). Other characters used in defining genera and species include whether corona lobes are horizontally spreading or ascending from the staminal column and posses wing-like keels as in species of *Pachycarpus lineolatus, P. campanulatus* and *P. bisacculatus* or not spreading from the staminal column as in *Glossostelma* (Goyder 1995); whether the proximal end of the corona reaches only as far as the base or middle of the column as in *Pachycarpus bisacculatus*, the top of the column as in *Pachycarpus lineolatus* (Goyder 1998a) or slightly taller than the column as in species of *Gomphocarpus* (Goyder and Nicholas 2001). Species of *Pachycarpus* and *Glossostelma* have dorsiventrally flattened corona lobes whereas in many species of *Gomphocarpus* they are laterally compressed. Species of Trachycalymma have mostly papilose (e.g. *Trachycalymma buchwaldii* and *T. foliosum*) or shortly fimbriate (e.g. *T. pseudofimbriatum*) corona lobes. Many species of *Miraglossum* and *Aspidoglossum* posses lobes with an apical process that can be long and curved clockwise from above and overlapping their neighbor forming

a wreath above the gynostegium as in *Miraglossum verticillare* or simply erect as in *Miraglossum pilosum* and *Aspidoglossum heterophyllum*. The position of attachment of the corona lobes to the column has also been used in generic and species delimitations. In *Trachycalymma pseudofimbriatum* and *Pachycarpus spurius* corona lobes arise from the base of the staminal column whereas many species of *Gomphocarpus* they arise from above base of staminal column.

8. **Association with sandy or rocky soils**— mostly inhabiting well drained sandy or rocky soils (0) mostly growing in neither sandy nor rocky soils (1)

The species of the African *Asclepias* complex have been reported in the literature as being found in a number of habitats including seasonally waterlogged grasslands, regularly burned highland or montane grasslands, open deciduous grasslands, highveild grasslands, mistbelt rocky grassland, *Themeda* coastal grassland, grassy slopes, *Brachystegia* woodlands, open *Brachystegia/Uapaca* woodland, mixed deciduous woodland, scrub or riparian woodland, open rocky ground, open *Protea* scrub, stoney hillsides, dry fallow land and disturbed areas such as alongside highways, bridges and railway lines. Some species have been reported in extremely arid environments such as the Namib desert and the Karoo, but apparently following dry water courses in some areas (Goyder and Nicholas 2001). Coding these areas posses a challenge due to the high number of combinations required to accommodate all the sampled species which are frequently found in various combinations of the habits above.

In all these different habitats, these plants are usually associated with sand, loam or black peaty soils, rocky or limestone ground or various combinations of these. Species have been reported as occurring in such specific substrates as black peaty soils, well drained soils, sandy soils, sand-loam soils, granite kopjes, rocky Hillsides, rock crevices, river banks and on dry shores of lakes or rivers. Sandy soils or rocky soils tend to be well drained soils which justifies treating here in one category. On the other hand non sandy or rocky soils tend to be less well drained and are therefore coded as the second state. However, this kind of coding used here may present some potential problems in that some of the species found in habits such as seasonally waterlogged grasslands where the exact type of soil is not specified and where they may actually be associated micro habits of sand. In cases where it was not clear whether there was an association of a species with sandy or rocky soils, they were coded as missing information.

RESULTS AND DISCUSSION

The morphological characters optimized on the phylogenetic tree were all homoplasious as indicated by multiple origins of many character states. The surprising parallel of many characters in some species suggests a high propensity for convergent evolution especially in morphological characters in the African *Asclepias* complex. Previous researchers have recognized that there are no exclusive synapormophies for most of the genera, which has led to the use of suites of characters to delimit genera (e.g. Goyder 1995; Goyder 1998a). Reconstruction of evolutionary patterns for floral and vegetative characters is important for understanding the taxonomic significance of these characters.

Stem branching— the branched stems state has been reconstructed as the ancestral condition (PP 0.91, table 8) in the African *Asclepias* complex and loss of branching has occurring at least several times (PP 0.91, table 8, Fig. 10, lineages A, B, C & D). Whether stems are unbranched or branched does not seem to be an exclusive occurrence in any resolved clade or in any of the putative genera. Even though there is not a complete correspondence, it appears that shifts from non branching to sometimes branching condition have only taken place in species with non reflexed corollas. This may suggest that this might be a way of increasing presentation of floral parts to pollinators because not having a strongly reflexed corolla seems to represent lesser exposure of the gynostegium to pollinators than the reflexed state.

Leaf type— Broad leaves are the plesiomorphic condition (PP 0.93, table 8) with narrow leaves being derived only a few (one or two times among sampled species) times, though this hypothesis of evolution is not very strongly supported (PP 0.73, table 8). There have been multiple reversals towards the broad leaved condition (PP 0.80, table 8; Fig.11, within clades A, B, C & D). In certain species reversals have occured to multi-condition states (both broad and narrow leaves occuring in one species). This aspect of leaf evolution does not seem to correspond with any single habitat type or geographic distribution. However, to some extent, leaf size seems potentially important in indicating relationships. For example as in the case of clade E (Fig. 11), which is a clade of broad leaved species of *Gomphocarpus* and the broad leaved *Pachycarpus* species pair within clade D (Fig. 11). This evidence supports Goyder et al's (2007) suggestion that vegetative characters might be a reliable guide to evolutionary relationships.

Inflorescence Position—Presence of nodal only inflorescences is the plesiomorphic condition (PP 0.96, table 8) within the African Asclepiadinae and possession of either nodal and terminal inflorescences on the same plant or terminal only inflorescences are derived states that were reconstructed as arising multiple times from nodal only ancestors (PP 0.83, table 8; Fig. 12, within clades A, B and C). There are three instances of 'nodal plus terminal' condition evolving from the nodal only condition and one instance of the terminal only condition evolving from the nodal and terminal condition. However it is not clear whether there is a single or multiple directions of evolution for the three states due to equivocal reconstruction of some intermediate nodes. Nodal inflorescences in milkweeds are extra axillary which are essentially terminal inflorescences whose terminal status has been superseded by new terminal inflorescences due to successive continued branching events from the terminal nodes. This scenario could arise when for example early onset of flowering is advantageous for a plant to be in synchrony with the presence of effective pollinators for reproduction or before onset of some undesirable condition such as excessive aridity or before the end of a short growing season. Terminal branching only could result from cessation of vegetative growth on a stem upon flowering. A possible scenario for this condition could be where vegetative development is initiated much earlier than the time of occurrence of pollinators and flowering needs to be delayed until the appropriate time leading to selection of late flowering individuals over time. Such changes in timing in the different flower positions could easily be achieved by dissociation of vegetative from reproductive development leading to different timings of the developmental processes (Diggle 1999).

Inflorescence attachment to the stem— Most species posses pedunculate inflorescences, whereas sessile inflorescence state may have arisen only a few times (PP 0.99, Table 8; Fig. 13) and may therefore be a good synapormophy for the *Aspidoglossum-Miraglossum* group (Clade A, Fig 13) with the exclusion of *Aspidoglossum heterophyllum.*

Orientation of petals— Within the African *Asclepias* complex, different types of non-reflexed petals were reconstructed as evolving independently multiple times from reflexed petals (Fig 14). However, this hypothesis of petal evolution was poorly supported (PP > 0.5, table 8). It was not clear what the ancestral condition was in the African *Asclepias* complex due to the equivocal reconstruction of the most basal node. There is one clear instance of a campanulate corolla arising from a spreading corolla.

Shape of the corona lobes— Corona shape was the most challenging character to code due to the high diversity of forms and the difficulty in determining the homology of different corona parts. States for this character were consequently coded as missing for many species. However the available results (Fig. 15), suggest that the *Gomphocarpus* type cucullate and laterally flattened corona lobes has given rise to dorsiventrally flattened forms and other cucullate forms (PP 0.75, table 8). This result seems to support Ollerton et al.'s (2003) suggestion that floral structures may in large part reflect phylogenetic relatedness rather than pollinator associations. However, since the corona acts as one of the structures that play an important role in guiding pollinators to the entrance of the anther slits for pollination (Kunze 1991), the diversity in form might be in part an effect of plant-pollinators interactions and this needs further investigation.

Association with sandy or rocky soils— In their various habitats, species of the African *Asclepias* complex are either associated with sandy, rocky, clay or loam soils (Fig. 16). Generally sandy or rocky soils imply well drained situations relatively more penetrable by roots and richer in essential gasses than other types of soil. Most species are associated with sandy or rocky soils which are the ancestral type of substrate (PP 1.00), table 8). Species have evolved into non sandy/rocky substrates (presumably less well drained soils) several times and most (12 out of 16) species associated with sandy or rocky soils are found either only in the eastern African center of diversity and adjacent areas or are widespread suggesting that the type of substrate may have also played a role in the diversification of the group.

The analysis of character evolution shows a propensity of most characters to evolve convergently probably as a result of adaptation to similar ecological pressures. Even though there is not complete correspondence, many species showing an association with non sandy soils or non rocky, tend to be broad leaved species and this current result (Fig 17), suggest that these species may have diversified into the broad leaved lineages as they colonized non sandy and non rocky (less well drained) habitats.

Phylogenetic uncertainty and Mapping uncertainty in character reconstruction -- The reconstructions inferred in this study are a good indication of trends in the evolution of the characters examined for the African *Asclepias* complex. However there are a number of factors to consider in interpreting these results. Reconstructing ancestral character states has always posed a challenge due to problems such as phylogenetic and mapping uncertainty (Ronquist 2004).

In this study, maximum parsimony reconstruction was used to reconstruct ancestral characters in this study. The MP method of reconstructing ancestral states like other methods, assumes adequate sampling of relevant taxa, correct coding of characters and that the tree being used is true. To check the effect of using only one tree (ML phylogeney) on the evolutionary trends reconstructed three diferent Bayesian trees were used to reconstruct all the characters and there was not difference in the general evolutionary trends reconstructed for all the characters.

There are many unsampled species of the African *Asclepias* complex, whose inclusion may at least slightly modify the character evolution trends inferred in this analysis. Furthermore, outgroups are also useful for indicating trends in character evolution and for them to be useful in this regard requires that they must not have themselves experienced those trends (Oakley and Cunningham 2000). However, it is usually not feasible, as in this study, to sample all the possible outgroup species so as to ensure inclusion of those outgroups that have not been influenced by the evolutionary trends in question within the ingroup. There is therefore a chance that the sampled outgroups may not be adequate or some of them may not be useful in inferring character evolution trends within the ingroup.

Since parsimony reconstruction is based on finding the fewest number of changes, there can be multiple equally parsimonious reconstructions of character changes, resulting in character reconstructions in certain parts of the tree being optimized as unequivocal. Parsimony also ignores all information about branch lengths due to its assumption that changes on all branches are equally likely. Methods like maximum likelihood reconstruction of ancestral characters account for branch

lengths and sometimes produce different reconstructions (Schluter et al. 1997). These should therefore be explored in future reconstructions. Simple parsimony also assumes that the rate of evolutionary character change is slow enough to allow recovery of evolutionary history (Omland 1999). Even though rapid divergence has not been explicitly shown for the African *Asclepias* complex phylogeny (Chapter 1 and 2), the short internal branches suggest a possibility of an episode of rapid lineages divergence having occurred in the group.

Another challenge usually considered in ancestral character reconstruction is that some biological basis of characters may need to be incorporated such as whether characters are possibly reversible or not (Cunningham et al. 1998) which might require incorporation of transformation weights. Therefore, a good understanding of the biology of the characters can lead to more reliable ancestral state reconstructions (Omland 1999). In the present study, there seems to be no apparent basis to expect any of the characters to be irreversible for them to require different transformation weights.

This study has reconstructed general trends in the evolution of a number of morphological and one habit character. The Sessile inflorescence condition may be a good synapomorphy for the Miraglossum-Aspidoglossum clade. The analysis of character evolution shows a tendency for most of these characters to evolve convergently probably as a result of adapting to similar ecological pressures. The non-reflexed petals within the African *Asclepias* complex may have evolved multiple times from reflexed petals even though this hypothesis requires further testing with increased species sampling and a more robust phylogenetic hypothesis. However, the

results suggest that stem branching is the ancestral condition in the African *Asclepias* complex and that loss of branching has arisen several times. Broad leaves have been reconstructed as the plesiomorphic condition with narrow leaves being derived only a few times and showing multiple reversals towards the broad leaved condition.

TABLE 7: Data matrix of six morphological characters and one habitat character

Taxa	**Characters**
	1234567
Cynanchum_ligulatum	?00???1
Pergularia_daemia	0015?01
Calotropis_procera	0013711
Kanahia_laniflora	0103?01
Trachycalymma_pseudofimbriatum	1005411
Asclepias_barjoniaefolia	00008?1
Asclepias_candida	?0?0??1
Asclepias_viridis	2013??1
Asclepias_davisii	0020?01
Asclepias_similis	10108?1
Asclepias_syriaca	2010811
Asclepias_oenotheroides	20008?1
Asclepias_stenophylla	10038?1
Asclepias_gibba	0123?01
Asclepias_macropus	1020??1
Asclepias_praemorsa	2113?01
Asclepias_randii	011??01
Asclepias_aurea	0113?01
Asclepias_cucullata	2113?01
Aspidoglossum_breve	1105610
Aspidoglossum_angustissimum	2103610
Aspidoglossum_delagoense	2103600
Aspidoglossum_ovalifolium	1203610
Aspidoglossum_heterophyllum	0203600
Glossostelma_spathulatum	1001311
Gomphocarpus_decipiens	0100101
Gomp_fruticosus_rostratus	0100101
Gomphocarpus_fruticosus	0100101
Gomphocarpus_physocarpus	0100111
Gomphocarpus_abyssinicus	0100101
Gomphocarpus_glucophyllus	1000111
Gomphocarpus_cancellatus	0000?01
Gomphocarpus_sostitialis	??2???1
Gomphocarpus_swynertonii	1000111
Gomphocarpus_stenophyllus	0100101
Gomphocarpus_integer	0100101
Gomphocarpus_filiformis	0101101
Gomphocarpus_praticolus	1000101
Gomphocarpus_tenuifolius	0100101
Margaretta_rosea	2114?11
Stathmostelma_pedunculatum	2113211
Xysmalobium_undulatum	1025?11
Pachycarpus_bissaculatus	2013011
Pachycarpus_goetzei	1015011
Pachycarpus_campanulatus_var._sutherlandi	2125011
Pachycarpus_lineolatus	2213011
Pachycarpus_petherikianus	1011011
Stenostelma_capense	0113??1
Miraglossum_pilosum	1110500
Miraglossum_verticillare	1100500
Asclepias_crispa	0110?01

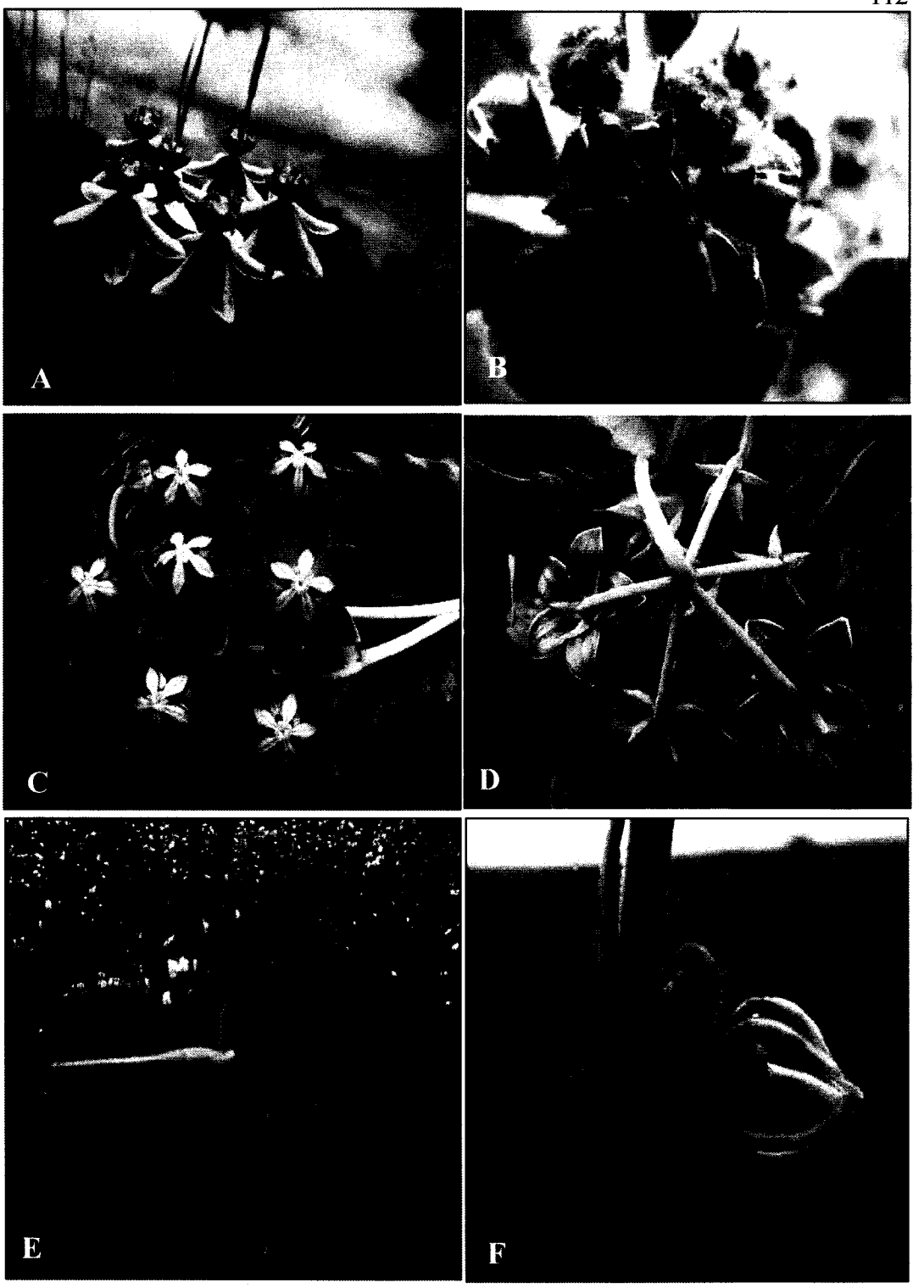

Fig. 9: Example genera of the African Asclepiadinae: A, *Stathmostelma pauciflorum* (Tanzania); B, *Pachycarpus petherickianus* (Tanzania); C & D, *Asclepias fulva*

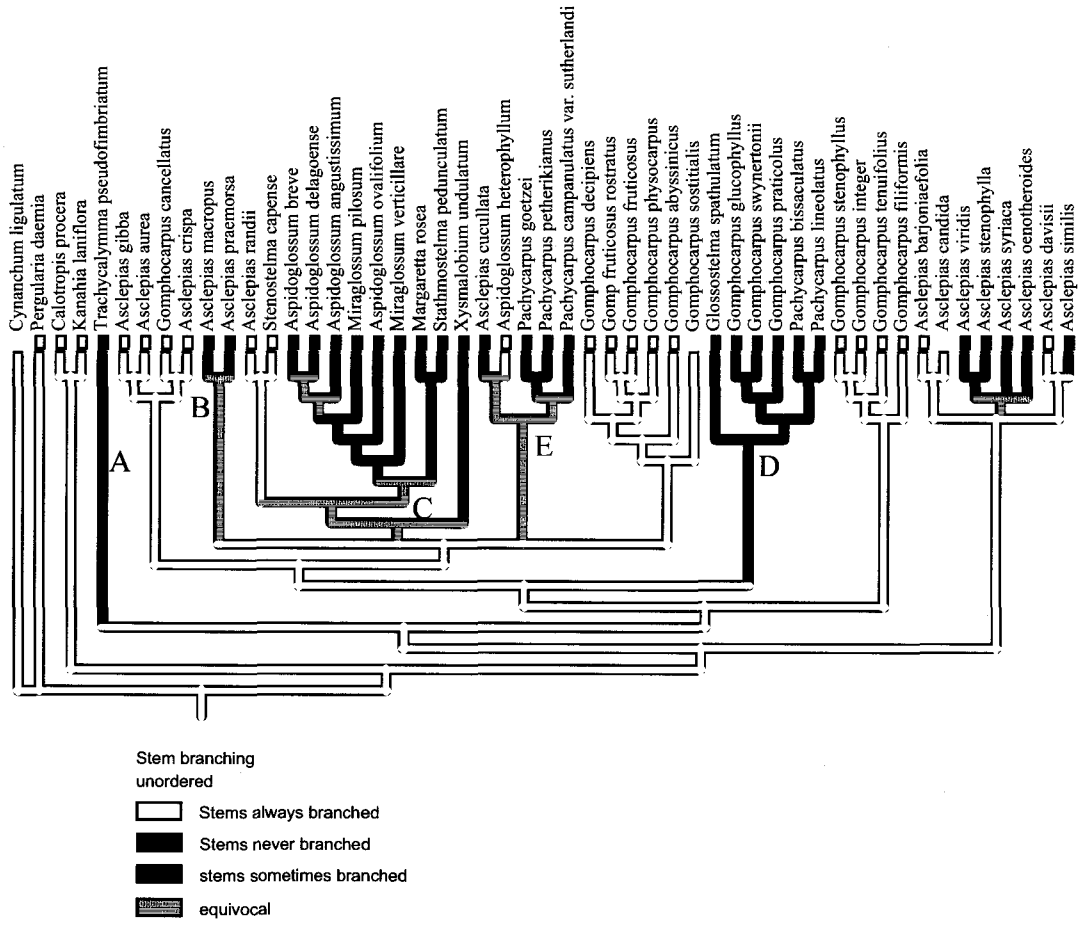

Fig. 10 Maximum parsimony optimization of Stem branching character states on a Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

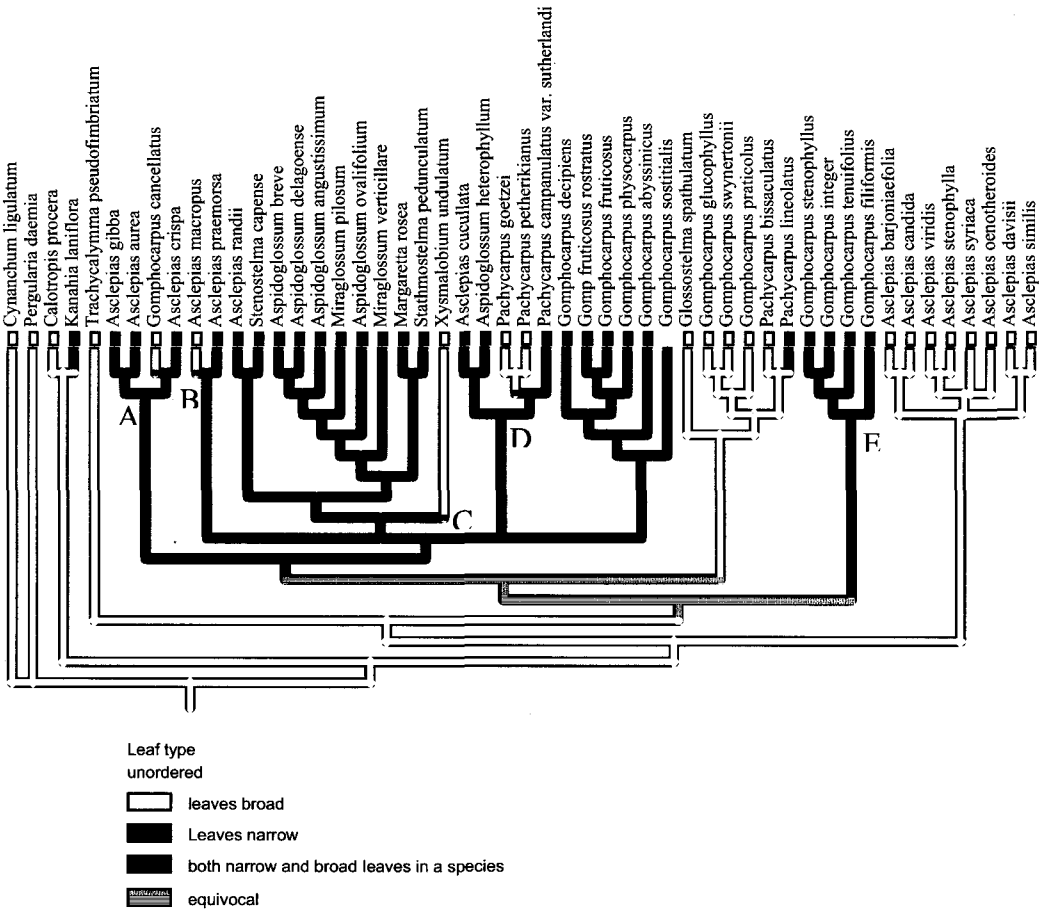

Fig. 11 Maximum parsimony optimization of Leaf type (relative width to length size) character states on a Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

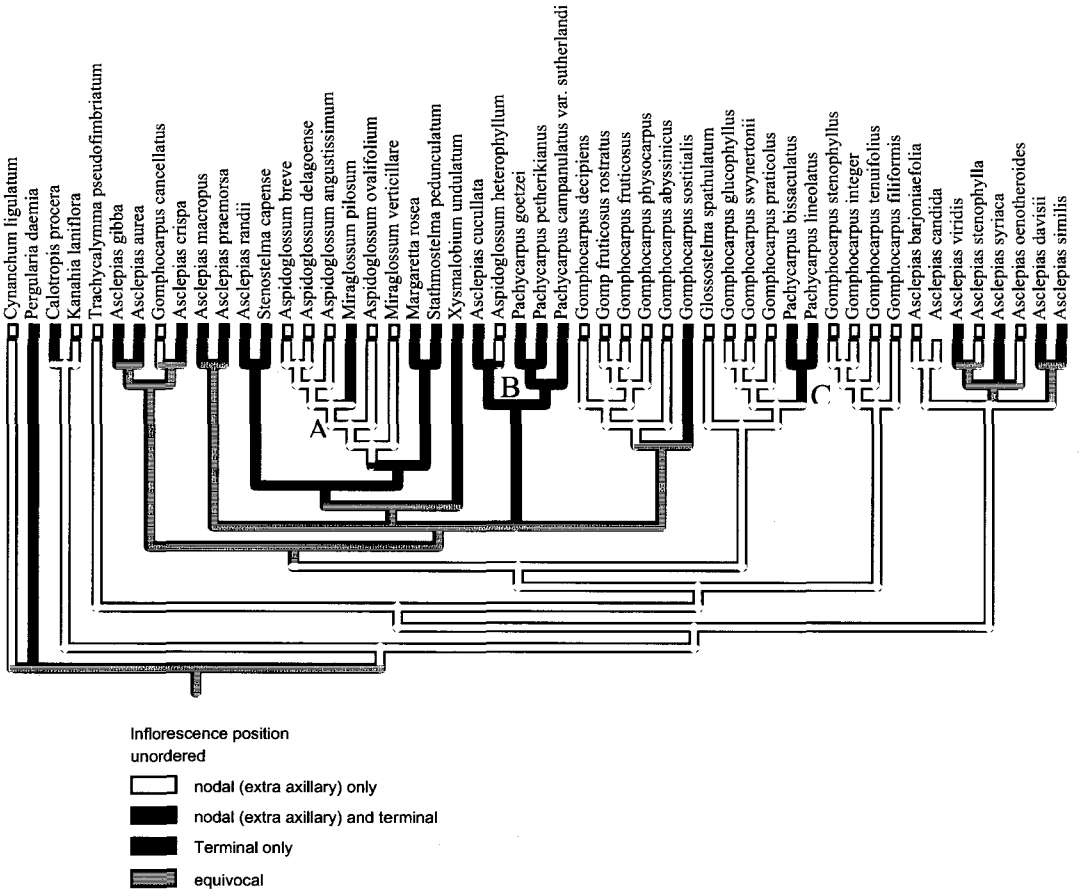

Fig. 12 Maximum parsimony optimization of inflorescence position character states on a Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

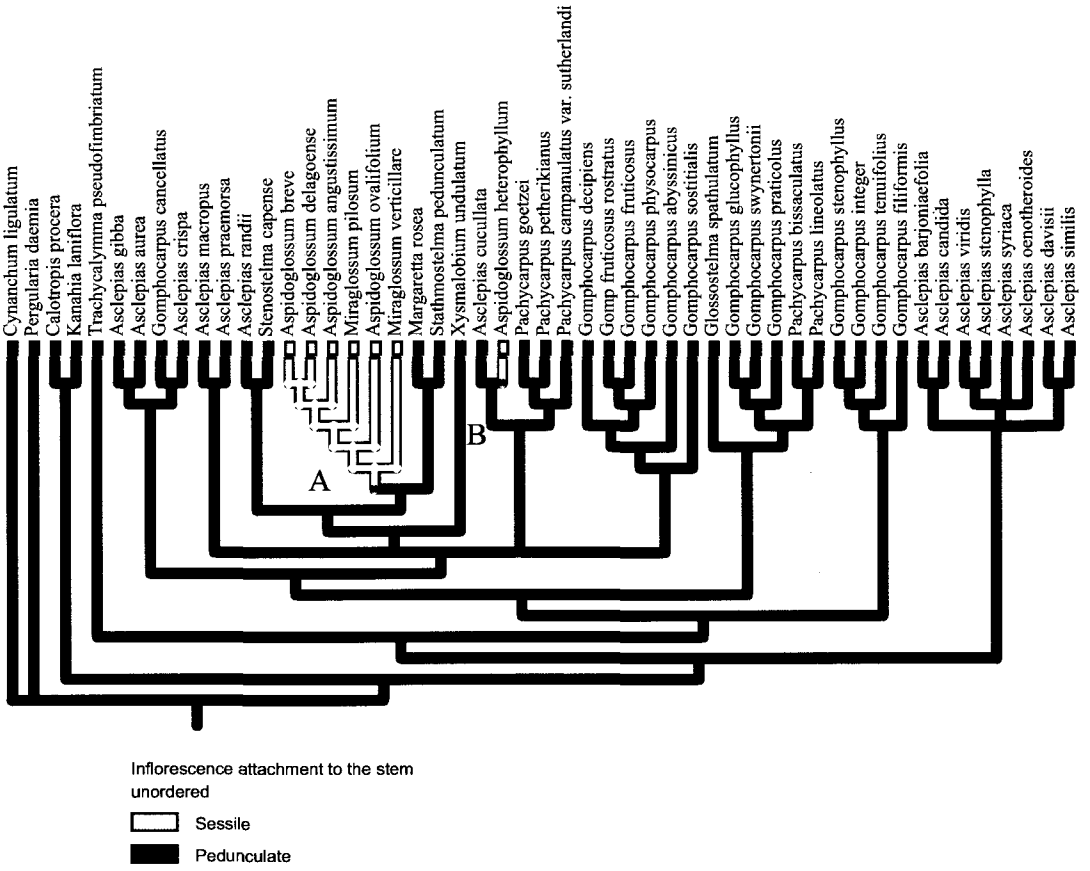

Fig. 13 Maximum parsimony optimization of inflorescence attachment character states on a Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

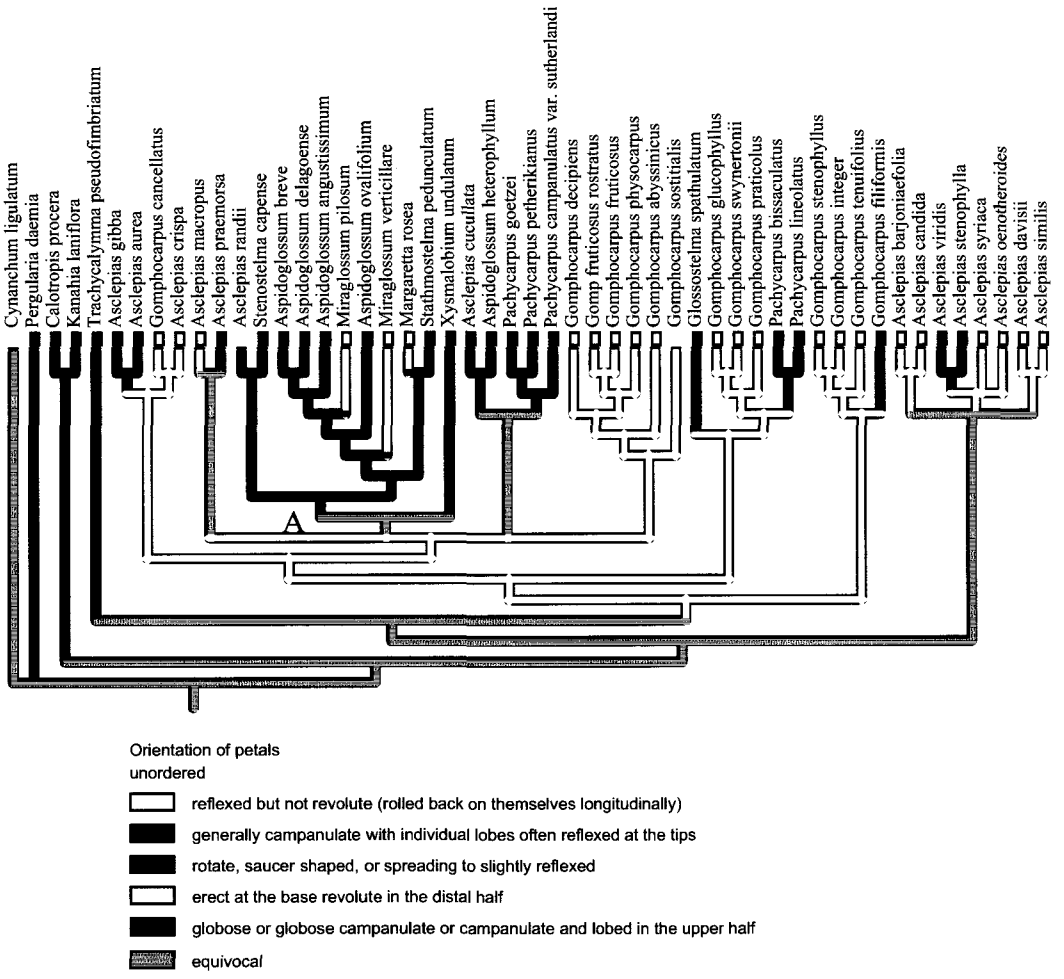

Fig. 14 Maximum parsimony optimization of petal orientation character states on a Maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

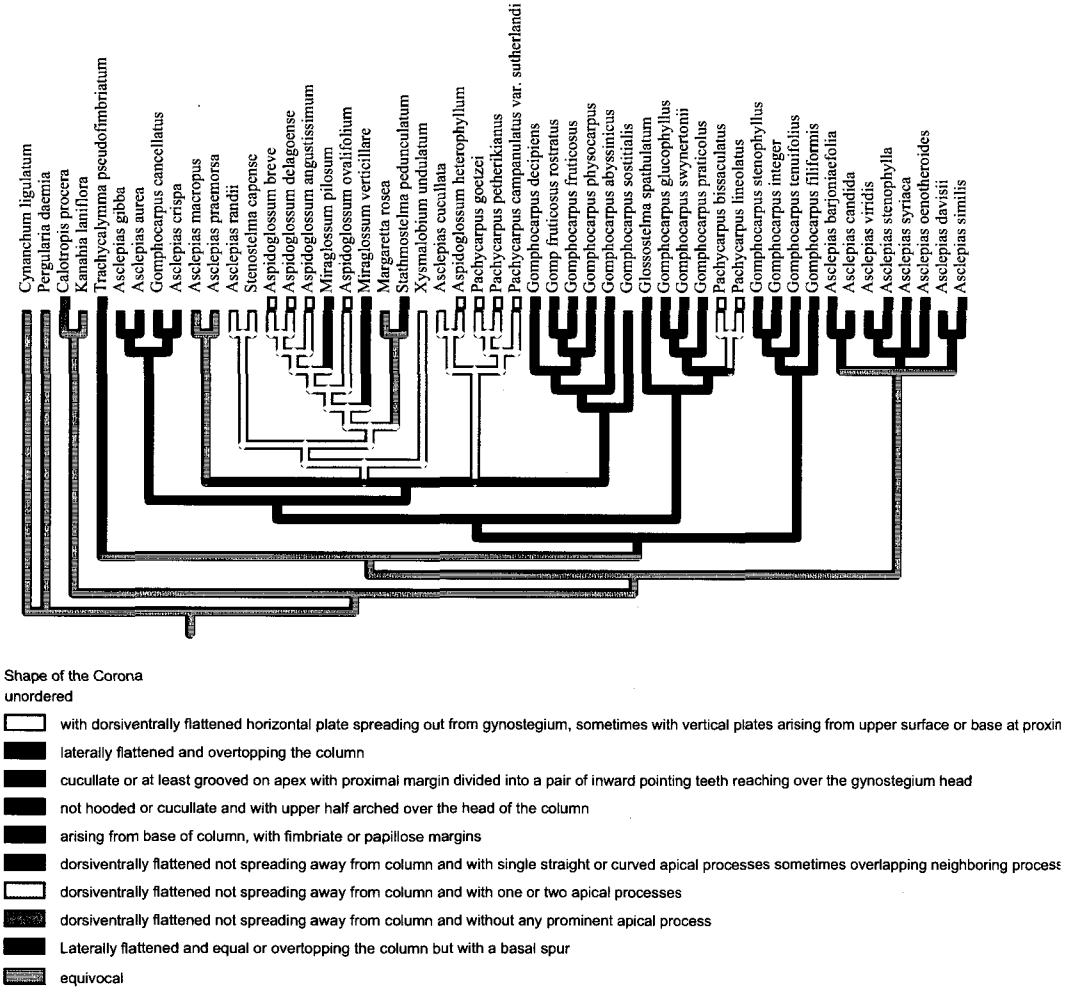

Fig. 15 Maximum parsimony optimization of corona shape character states on a maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

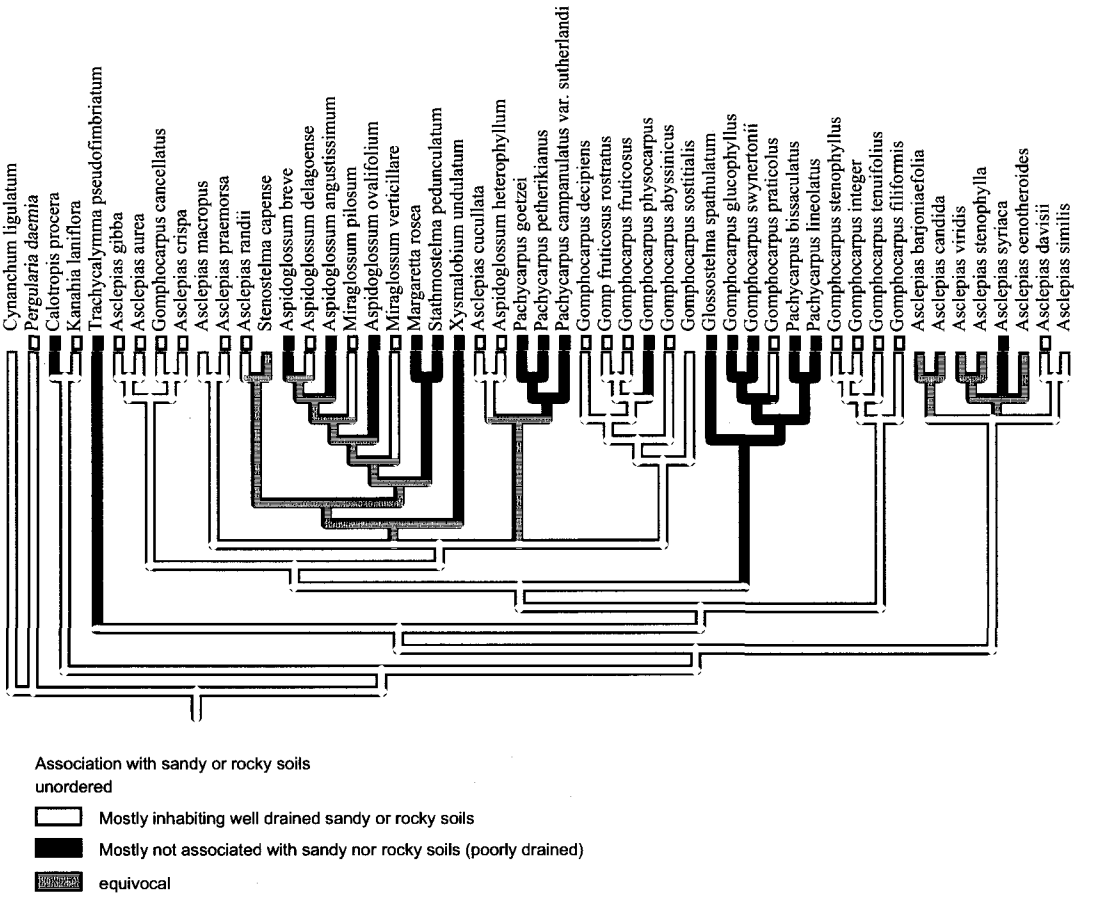

Fig. 16 Maximum parsimony optimization of species association with sandy or rocky soils character states on a maximum likelihood tree of the African *Asclepias* generic complex inferred by ML ratchet method from combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

Fig. 17

Association with sandy or rocky soils

- mostly inhabiting well drained sandy or rocky soils
- mostly growing in neither sandy nor rocky soils
- Missing information

Leaf type

- leaves broad
- leaves narrow
- both narrow and broad leaves in a species

Fig. 17 Maximum parsimony optimization leaf type, with species association with sandy or rocky soils indicated by data boxes. The leaf type was traced on a tree inferred by maximum likelihood analysis of combined sequences of *rpl16*, *trnC-rpoB*, *trnS-G/trnG* cpDNA regions and partial sequences of the low copy nuclear *PgiC* gene.

Table 8: Support for various hypotheses of character evolution that were reconstructed by maximum parsimony method. The support is based on a manual examination of 100 randomly selected Bayesian trees to check for ancestral character reconstruction supporting the proposed hypotheses.

CHARACTER	HYPOTHESIS	PERCENTAGE OF BAYESIAN TREES SUPPORTING THE HYPOTHESIS
Stem branching		
	branched stems are ancestral	91%
	Stem branching lost several times	91%
Leaf type		
	Broad leaved state plesiomorphic	93%
	narrow leaves derived only a few (one or two) times among sampled species	73%
	multiple reversals towards the broad leaved condition	80%
Inflorescence Position		
	Presence of nodal only inflorescences is the plesiomorphic condition	96%
	either nodal and terminal inflorescences on the same plant or terminal only inflorescences are derived states that were reconstructed as arising multiple times from nodal only ancestors	83%
Inflorescence attachment to the stem		
	sessile inflorescence state arising only a few (one or two) times among sampled species, from pedunculate inflorescences	99%
Orientation of petals		
	non-reflexed type petals evolving independently multiple times from reflexed petals within the African Asclepias complex	47%
Shape of the corona lobes		

	Gomphocarpus type cucullate and laterally flattened corona lobes giving rise to dorsiventrally flattened forms and other cucullate forms	75%
Association with sandy or rocky soils		
	Species have evolved into non sandy/rocky substrates (less well drained soils) several times	100%

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Appendix A

Species, locality data and voucher information for taxa sampled in the study. Species names in quotation marks are taxa that where excluded from their respective genera but not yet placed in other genera.

TAXON	Locality	Voucher information
Stathmostelma gigantiflorum K.Schum.	Kenya: Machakos District	Havey and Vollesen 64 [K]
Stathmostelma spectabile (N.E.Br.) Schltr. subsp. *frommii* (Schltr.) Goyder	Tanzania: Nkasi District	S.Bidgood et al. 2597 [K]
"Stathmostelma verdickii De Wild.*" (see putative affinities)*	Tanzania: T4	S.Bidgood et al. 2647 [K]
Stathmostelma welwitschii Britt.& Rendl.	Tanzania: Sumbawanga District	S.Bidgood et al. 2403 [K]
Stathmostelma spectabile (N.E.Br.) Schltr. ssp *spectabile*	Zambia: Balmoral Ranch	Bingham & Truluck 10279 [K]
Stathmostelma pedunculatum (Decne.) K.Schum.	Kenya: Shimba Hills	Harvey & Vollesen 51 [K]
Stathmostelma pauciflorum (Klotzsch) K.Schum.	Kenya: Kwale District	Luke and Gray 4030 [K]
Stathmostelma fornicatum (N.E.Br.) Bullock subsp. *tridentatum* Goyder	Tanzanya: Mbeya District	T.C.E. Congdon 319 [K]
Margaretta rosea Oliv. subsp. *corallina* Goyder	Tanzania: T4. Ufipa District: 4 km W of Mkundi on road to Kamwanga	D.J. Goyder et al. 3791 [K]
Pachycarpus lineolatus (Decne.) Bullock	Tanzania: T4, Ufipa District	Gereau 6290 [MO]
Pachycarpus campanulatus (Harv.) N.E.Br. var.	South Africa: Eastern Cape	Bester 3395 [K]

sutherlandii N.E.Br.		
Pachycarpus bisacculatus (Oliv.) Goyder	Tanzania: T6	Bidgood, S.; Vollesen, K.; et al 418 [K]
Pachycarpus goetzei (K.Schum.) Bullock	Tanzania: T4: Sumbawanga District	S. Bidgood et al. (2460) 10901 [J]
Pachycarpus petherickianus (Oliv.) Goyder	Kenya: West Suk district, Keringet-Lodwar road	Tweedie (3804) 10902 [J]
Pachycarpus spurius (N.E.Br.) Bullock	Tanzania: T4. Ufipa District west of Mkowe on Chapotaroad	Goyder, D.J.; et al 3781 [K]
Glossostelma carsonii (N.E.Br.) Bullock	Malawi, Northern Region	Phillips 4166B [MO]
Glossostelma ceciliae (N.E.Br.) Goyder	Tanzania: T4. Ufipa District	Goyder, D.J.; et al 3834 [K]
Glossostelma spathulatum (K.Schum.) Bullock	Zimbabwe	Goyder, D.J. and A. Paton 4108 [K]
Xysmalobium undulatum (L.) W.T.Aiton	Johannesburg, South Africa	K. Balkwill 10846 [J]
Xysmalobium fraternum N.E.Br.	Tanzania: T4: Sumbawanga District	S. Bidgood et al., 2390 [K]
Xysmalobium heudelotianum Decne.	Zambia, North Luangwa National Park,	Smith 103 [K]
Xysmalobium involucratum (E.Mey.) Decne.	South Africa, Cape Province, Maclear.	S. P. Bester (3418) 11366 [LHMS]
Xysmalobium stocksii (N.E. Br.) Bullock	Kenya: Kwale District, Shimba Hills	Luke 30988 [J]
Xysmalobium parviflorum Harv. ex Scott-Elliot	South Africa, Natal	Killick & Vahrmeijer 3658 [10631 LHMS]
Xysmalobium acerateoides (Schltr.) N.E.Br.	South Africa, Transvaal	W.N. & K. Ellery 92/141 [10629 LHMS]
Gomphocarpus abyssinicus Decne.	Ethiopia, Welga west of Mendi	Friss 7832 [K, C]
Gomphocarpus filiformis (E.Mey.) D.Dietr.	S.Africa, Namibia (Southern Africa)	Kubriske 56 [J]
Gomphocarpus integer (N.E. Br.) Bullock	Kenya: K4, Nairobi District, Athi River	Goyder et al., 3713 [K]
Gomphocarpus physocarpus E.Mey.	South Africa, Johannesburg	Balkwill 10842 [J]
Gomphocarpus stenophyllus Oliv.	Ethiopia, Gamu Gofa 38km from Jinka on Koso road	Gilbert & Phillips 9025 [K]

Gomphocarpus fruticosus (L.) W.T.Aiton subsp. *fruticosus*	Tanzanya: Mbeya District, see Goyder & Nicholas 2001 for dist. map	Lambinon 95/493 [ARIZ]
Gomphocarpus fruticosus (L.) W.T.Aiton subsp. *rostratus* (N.E.Br.) Goyder & Nicholas	Botswana	D.G. Long & D.A.H. Rae 491 [K]
Gomphocarpus cancellatus (Burm.f.) Bruyns	South Africa	P.B. Drewe 534 [K]
Gomphocarpus fruticosus (L.) W.T.Aiton subsp. *decipiens* (N.E.Br.) Goyder & Nicholas	South Africa, Johannesburg	Balkwill 10843 [J]
"*Gomphocarpus solstitialis*" (A.Chev.) Bullock	Ivory Coast: Marahoue National Park	Jongkind & Diomaudé 4618 (K)
Gomphocarpus glucophyllus Schltr.	Zambia	A Strid 2266 [no data]
Gomphocarpus tomentosus Burch.	South Africa, Transvaal	Stalmans 430 [no data]
Gomphocarpus munonquensis (S.Moore) Goyder & Nicholas	Tanzania (Mpanda District Kampisa sitebi)	T.C.E. Congdon 501[K]
Gomphocarpus swynnertonii (S.Moore) Goyder & Nicholas	Tanzania T4	Goyder 3805 [K]
Gomphocarpus kaessneri (N.E.Br.) Goyder & Nicholas	Tanzania	Grimshaw 9496 [no data]
Gomphocarpus praticola (S.Moore) Goyder & Nicholas	Tanzania T4, Ufipa district 2km from Sumbawanga, on Mbisi road	Goyder 3812 [K]
Gomphocarpus semilunatus A.Rich.	Kenya K3, Naivashia Yatch club	Goyder 3710 [K, EA, PRE, WAG]
Gomphocarpus tenuifolius (N.E.Br.) Bullock	Zimbabwe	Seine 648 [no data]
Trachycalymma buchwaldii (Schltr. & K. Schum.) Goyder	Tanzania: Iringa District, Mount Image	Goyder 3924 [DSM, K, PRE]
Trachycalymma foliosum(K.Schum.) Goyder	Tanzania: Iringa District	de Leyser 305 [K]

*Trachycalymma pseudofimbriatum*Goyder	Ethiopia, Sidamo Province, Agere Selam	Haugen 1503 [K]
Trachycalymma pulchellum (Decne.) Bullock	Zaire: Kikandaka	Malaisse 13730 [K]
Aspidoglossum breve Kupicha	Malawi: Nyika National Park	Goyder et al. 3557 [K]
Aspidoglossum heterophyllum E.Mey.	South Africa, Western Cape, Napier	P. Bruyns 6855 [3293 LHMS]
Aspidoglossum connatum (N.E.Br.)Bullock	Mbala, Zambia	DC280 [HPSU]
Aspidoglossum delagoense (Schltr.) Kupicha	Mozambique: Maputo	Goyder 5006 [K]
Aspidoglossum ovalifolium (Schltr.) Kupicha	South Africa, Johannesburg	Balkwill [10546 LHMS]
Aspidoglossum angustissimum (K.Schum.) Bullock	Malawi: Nyika Plateau	Patel et al. 5116 (K)
Aspidoglossum interruptum E.Mey.) Bullock	Zambia: North Luangwa Nat. Park	Smith 428 (K)
Aspidonepsis flava (N.E.Br.) Nicholas & Goyder	South Africa: Eastern Cape	Bester 3373 (K)
Stenostelma corniculatum (E. Mey.) Bullock	South Africa	Balkwill 10908 [J]
Schizoglossum hamatum E. Mey.	South Africa: Eastern Cape	Bester 3509 [no data]
Schizoglossum alpestre K. Schum	Tanzania: T7, Njombe District	D.J.Goyder et al. 3892 [K]
Schizoglossum hillardiae Kupicha	South Africa: KwaZulu-Natal	Edwards 1766 [K]
Asclepias praemorsa Schltr.	South Africa, Transkei	Nicholas & Smook 2388 [10620 LHMS]
Asclepias gibba (E.Mey.) Schltr. var gibba	South Africa, Makhalaneng	Hargreaves 3483 [K]
Asclepias aurea (Schltr.) Schltr.	South Africa, Transvaal, Lydenberg	G. Germishuizen 444 [K]
Asclepias randii S. Moore	Tanzania, Mbeya District	D.J. Goyder et al. 3854 [K]
Asclepias macropus (Schltr.) Schltr.	South Africa, Cape Province	S.P. Bester 3582 [K]
Asclepias densiflora N.E.Br.	South Africa, Johannesburg	K. Balkwill 10844 [J]
Asclepias disparilis N.E.Br.	South Africa, Natal	Hilliard & Burtt 16705
Asclepias cultriformis	South Africa, Natal	J.H. Ross 1876[K]

(Harv. ex Schltr.) Schltr.		
Asclepias cucullata (Schltr.) Schltr.	South Africa: Eastern Cape	Bester 3209 (K)
Asclepias multicaulis (E.Mey.) Schltr.	South Africa, Transkei	OH Hilliard & B.L. Burtt 18708 [K]
Asclepias fulva N.E.Br.	Tanzania: Sumbawanga District	D. Chuba 280 [HPSU]
Gen. indet aff. *Asclepias*. (10488)	Tanzania, Njombe District	Goyder 3895 [K]
Gen. indet. aff. *Asclepias* (10917)	Obtained from Kew DNA bank	Richards 22589 [no data]
Cynanchum ligulatum (Benth.) Woodson	Mexico, Sonora	Fishbein 3581 [WS]
Pergularia daemia (Forssk.) Chiov.	Tanzania	Fishbein 5445 [MISSA]
Calotropis procera (Linn.) Ait.	USA, Arizona	Need Voucher
Kanahia laniflora (Forssk.) R.Br.	Tanzania, Iringa Dist.	D.J. Goyder et al. 3931[K]
Miraglossum verticillare (Schltr.) Kupicha	South Africa, KwaZulu Natal, Wahroonga Farm near Howick on the underberg Road (R617)	Adam Shuttleworth 2 [NU]
Miraglossum pilosum (Schltr.) Kupicha,	South Africa, KwaZulu Natal, Wahroonga Farm near Howick on the underberg Road (R617)	Adam Shuttleworth 3 [NU]
Stenostelma capense Schltr	Namibia, Kombat, 1917DA	P.V. Bruyns 10635 [NBG]
Asclepias crispa P.J.Bergius	South Africa, Olof Bergh Pass, 5Km south of Redelinghuys	PVB 10890a [NBG]
Asclepias amplexicaulis Sm.	USA, Wisconsin	Lynch 12652 [LSUS]
Asclepias asperula (Decne.) Woodson ssp. asperula	USA, Texas	Lynch 12014 [LSUS]
Asclepias candida Vell.	Bolivia, Santiago	Wood & Goyder 16919 [K]
Asclepias barjoniifolia E. Fourn.	Bolivia, Tarija	Wood 9532 [K]
Asclepias flava (Kuntze) Lillo, non N.E. Br.	Argentina	Zuloaga & Morrone 7215 [HPSU]
Asclepias connivens Baldwin ex Elliott	USA, Florida	Lynch 12336 [LSUS]
Asclepias longifolia Michx.	USA, Florida	Lynch 12447 [LSUS]

Asclepias purpurascens L.	USA, Ohio	Lynch 12847 [LSUS]
Asclepias syriaca L.	USA, Wisconsin	Lynch 12589 [LSUS]
Cynanchum ligulatum (Benth.) Woodson	Mexico, Sonora	Fishbein 3581 [WS]
Asclepias syriaca L.	USA, Wisconsin	Lynch 12589 [LSUS]
Asclepias similis Hemsl	Mexico, Querétaro	Fishbein 5148 [MISSA]
Asclepias viridis Walter	USA, Florida	Lynch 12491 [LSUS]
Asclepias stenophylla A. Gray	USA, Kansas	Fishbein 2817 [ARIZ]
Asclepias oenotheroides Schltdl. & Cham.	USA, Texas	Lynch 13339 [LSUS]
Asclepias davsii	USA	

Appendix B

Sources of information used in the coding of morphological characters. The sources include photographs of herbarium specimens with their voucher information, physical herbarium specimens with their voucher information, photographs taken in their field, photographs from internet websites and literature sources. All pictures of herbarium specimens where obtained from the Kew Herbarium catalogue (http://apps.kew.org/herbcat/navigator.do).

Taxon	Herbarium specimen Photographs voucher information	Herbarium specimen voucher information	Other photograph sources	Literature sources
Stathmostelma pedunculatum	Hildebrandt, J.M. 1901, Tanzania [K]	Lye 2000, Uganda [MHU]		(Goyder 1998b)
	Volkens 87, Tanzania [K]	Morrison s.n. Uganda [MHU]		
	Schimper s.n. Ethiopia [K]	Lind s.n. Uganda [MHU]		
	Hannington, J. s.n., Tanzania [K]	Wood 703, Uganda [MHU]		
		Lye 4563, Uganda [MHU]		
		Synnott 1578, Uganda [MHU]		
		Katende 1001, Uganda [MHU]		
		Lye 3505, Uganda [MHU]		
		Msenga 4, Uganda [MHU]		
		Miller 190, Uganda		

		[MHU]		
		Hamilton 49, Uganda [MHU]		
Margaretta rosea subsp. *corallina*				(Goyder 2005)
Pachycarpus lineolatus	Carson, A. 12 Zambia [K]	D. M. Nath Nair 21254, Zambia [UZL]		(Goyder 1998a; Smith 1988)
	Mildbraed 9318 Cameroon [K]	D. M. Nath Nair 20227, Zambia [UZL]		
	Schweinfurth , G. 1960 Sudan [K]			
	Mildbraed 9391 Cameroon [K]			
		Rwaburindore 5781, Uganda [MHU]		
Pachycarpus campanulatus var. *sutherlandii*	Gerrard, W.T. 1299 South Africa [K]			(Goyder 1998a; Smith 1988)
	Sutherland s.n. South Africa [K]			
	Gerrard 1298 South Africa [K]			
	Cooper, T. 2731 Lesotho [K]			
	Rehmann, A. 5871 South Africa [K]			
Pachycarpus bisacculatus	Johnston, H.H. s.n.			(Goyder 1998a; Smith

	Tanzania [K]			1988)
	Gossweiler 13891 Angola [K]			
	Kassner 2307 , Democratic Republic Congo [K]			
	Volkens, G. 2129 Tanzania [K]			
Pachycarpus goetzei	Bidgood, S.; Congdon,T.C .E. 154 Tanzania [K]	M. Richards 16117, Zambia [UZL]		(Goyder 1998a; Smith 1988)
	Whyte, A. 108 Malawi [K]			
	Whyte, A. 129 Malawi [K]			
Pachycarpus petherickianus			D. Chuba	(Goyder 1998a; Smith 1988)
Glossostelma spathulatum	Kirk, J. s.n. Malawi [K]	M. Richards 18840, Zambia [UZL]		(Goyder 1995)
	Buchanan, J. 603 Malawi [K]	M. Richards 15968, Zambia [UZL]		
	Buchanan, J. 43 Malawi [K]	M. Richards 18860, Zambia [UZL]		
	Mechow 539a Angola Quango [K]			
	Cameron, K.J. 12 Malawi [K]			
	Carson, A. 62 Zambia [K]			
Xysmalobium undulatum	Welwitsch 4170 Angola	C.C. Epukiro 2119, Namibia [WIND]		(Langley 1980)

	[K]			
	Meihow 329 Angola [K]			
	Baum s.n. Angola [K]			
	Welwitsch 4171 Angola [K]			
	Burchell, W.J. 2491 South Africa [K]			
	Burchell, W.J. 1834 South Africa [K]			
	Cameron, K.J. 4 Malawi [K]			
	Holub, E. 816 Botswana [K]			
	Scott Elliot, G.F. 6877 Kenya [K]			
	Holub, E. 669 Botswana [K]			
	Kirk, J. s.n. Malawi [K]			
	Giess W. 10666, S.W Africa [K]			
Gomphocarpus filiformis	Burke 247 South Africa [K]	Koberg H., Loots S., Moses K. NAM2331/HK1246, Namibia [WIND]		(Goyder and Nicholas 2001)
	Drege 892 South Africa [K]	Schinz, H. 18 Namibia [WIND]		
	Schinz, H. 20 Namibia [K]	Strohbach B. Kubirske et al 2940, Namibia [WIND]		
	Zeyher 1167 South	Smith G. SMI 3/9, Namibia [WIND]		

	Africa [K]			
	Bolus, H. 640 South Africa [K]			
	Schinz, H. 18 Namibia [WIND]			
	Strohbach B. Kubirske et al 2940, Namibia [WIND]			
	Smith G. SMI 3/9, Namibia [WIND]			
	Koberg H., Loots S., Moses K. NAM2331/H K1246, Namibia [WIND]			
Gomphocarpus integer	Johnston, H.H. s.n. Tanzania [K]	Lee 16, Uganda [MHU]		(Goyder and Nicholas 2001)
	Volkens, G. 567 Tanzania [K]			
	Cole, E. s.n. Somalia [K]			
	Lort Phillips, E. s.n. Somalia [K]			
	James; Thrupp s.n. Somalia [K]			
	Smith, C.S. s.n. Tanzania [K]			
	Lort Phillips, E. s.n. Somalia [K]			
	Drake Brockman;			

	Comm s.n British Somaliland [K]			
Gomphocarpus stenophyllus	Speke; Grant s.n. Tanzania [K]	Lind 3153, Uganda [MHU]		(Goyder and Nicholas 2001)
Gomphocarpus tenuifolius	Baines s.n. Zimbabwe [K]			(Goyder and Nicholas 2001)
Gomphocarpus cancellatus				(Goyder and Nicholas 2001)
	Burke s.n. South Africa [K]			
Gomphocarpus fruticosus subsp. *decipiens*	Conrath, P. 997 South Africa [K]			(Goyder and Nicholas 2001)
	Gerrard, W.T. 1290 South Africa [K]			
	Schlechter, F.R.R. 3589 South Africa [K]			
	Gilfillan, D.F. 6045 South Africa [K]			
	Leendertz, R. 607 South Africa [K]			
	Seygel R. 3436, S.W Africa [K]			
	le Roux J. 933, S.W			

	Africa			
	Immelman K.L.454,S.W Africa [K]			
	Muller M. & Kolberg H. 2091, S.W Africa [K]			
Gomphocarpus fruticosus subsp. *fruticosus*	Gilfillan, D.F. 6045 South Africa. [K]	B. de Winter 2411, Namibia [WIND]		(Goyder and Nicholas 2001)
	Leendertz, R. 607 South Africa [K]	R. Seydel 3436, Namibia [WIND]		
	Conrath, P. 997 South Africa [K]	A, Angus1409, Zambia [UZL]		
	Gerrard, W.T. 1290 South Africa [K]			
	Schlechter, F.R.R. 3589 South Africa [K]			
	Lort-Phillips, E. s.n. Somalia [K]			
	Cole, E. S.n. Somalia [K]			
	Baum, H. 500 Angola [K]			
Gomphocarpus fruticosus subsp. *rostratus*	Lugard, E.J. 22 Botswana [K]	J. Lerox 933, Namibia [WIND]		(Goyder and Nicholas 2001)
	Baum, H. 500 Angola [K]			

	Lugard, E.J. 231 Botswana [K]			
Gomphocarpus physocarpus	Glaziou 6706 Brazil [K]	Eggling 323, Uganda [MHU]		(Goyder and Nicholas 2001)
	Gerrard 511 South Africa [K]	Brown 133, Uganda [MHU]		
	Drege s.n. South Africa [K]	M. Richards 12027, Zambia [UZL]		
	Drege s.n. South Africa [K]	Lye 3459, Uganda [MHU]		
		Synnott 673, Uganda [MHU]		
		Wakabi 13, Uganda [MHU]		
		Mugasha 15, Uganda [MHU]		
		Kertland, 386, Uganda [MHU]		
		Wood 618, Uganda [MHU]		
		Lye 3117, Uganda [MHU]		
Gomphocarpus abyssinicus	Schimper 1674 Ethiopia [K]			(Goyder and Nicholas 2001)
	Schimper 503 Ethiopia [K]			
	Chevalier, A. 18372 Guinea [K]			
Gomphocarpus glaucophyllus	Galpin, E.E. 663 South Africa [K]	M. Richards 12717, Zambia [UZL]		(Goyder and Nicholas 2001)
	McLea, J.H. 3086 South Africa [K]	D. Vesey-FitzGerald 1324, Zambia [UZL]		
Gomphocarpus swynnertonii	Stolz, A. 105 Tanzania [K]			(Goyder and Nicholas 2001)

Gomphocarpus praticolus				(Goyder and Nicholas 2001)
Trachycalymma pseudofimbriatum	Haugen, T. 1503 Ethiopia [K]			(Goyder 2001)
Miraglossum verticillare	Fannin, G. (b) South Africa [K]		Peter Wragg, MG6109	(Kupicha 1984)
	Bolus, H. 3242 South Africa [K]			
	Wood, J.M. 316 South Africa [K]			
	Gerrard, W.T. 1307 South Africa [K]			
Miraglossum pilosum	Stewart J.2195 South Africa [K]		Peter Wragg, RA8922, RA8945	(Kupicha 1984)
Aspidoglossum breve	Hilliard, O.M.; Burtt, B.L 4399 Malawi [K]			(Kupicha 1984)
Aspidoglossum heterophyllum	Schlechter, F.R.R. 2387 South Africa [K]			(Kupicha 1984)
	Bolus, H. 4488 South Africa [K]			
	Drege s.n. South Africa [K]			
	Harvey, W.H. s.n. South Africa [K]			
	Prior, A. s.n. South Africa [K]			
	Bolus, H. 6695 South Africa [K]			

	South, B. 627 South Africa [K]			
	Brownlee, J. s.n. South Africa [K]			
	Zeyher 347 South Africa [K]			
	Barber 112 South Africa [K]			
	MacOwan, P. 660 South Africa [K]			
	Holland 38 South Africa [K]			
Aspidoglossum delagoense	Junod 184 Mozambique [K]			(Kupicha 1984)
Aspidoglossum ovalifolium	Gilfillan, E.E. 6229 South Africa [K]			(Kupicha 1984)
	Flanagan, H.G. 1307 South Africa [K]			
	Bolus, N. 11123 South Africa [K]			
	Gilfillan, D.F. 6230 South Africa [K]			
	Schlechter, F.R.R. 3496 South Africa [K]			
	Bolus, N. 12145 Swaziland [K]			
	Wood, J.M.			

	s.n. South Africa [K]			
	Fry, H. 2747 South Africa [K]			
	Wood, J.M. 316 South Africa [K]			
	Gueinzius s.n. South Africa [K]			
Aspidoglossum angustissimum	Schweinfurth, G. 3879 Sudan [K]	Lind, 2447, Uganda [MHU]		(Kupicha 1984)
	Schweinfurth, G. 3539 Sudan [K]			
	Schweinfurth, G. 3720 Sudan [K]			
	Whyte, A. 353 Malawi [K]			
	Chase, N.C. 6262 Zimbabwe Montali [K]			
	Lind, 2447, Uganda [MHU]			
Asclepias praemorsa	Drege 4971 South Africa [K].			(Nicholas 1982)
Asclepias gibba	Flanagan, H.G. 397 South Africa [K]		Swaziland's Flora Database: http://www.sntc.org.sz/flora/index.asp	(Nicholas 1982)
	Sole, M. 372 South Africa [K]			
	Drege 4967 South Africa [K]			
	Sanderson, J. 164 South Africa [K]			

	Drege 4973 South Africa [K]			
	Cooper, T. 2725 Lesotho [K]			
	Burtt-Davy, J. 795 South Africa [K]			
	Burke s.n. South Africa [K]			
	Leendertz, R. 328 South Africa [K]			
Asclepias macropus	Wood, J.M. 4544 South Africa [K]			(Nicholas 1982)
	Bowker s.n. South Africa [K]			
	Fannin, G. 90 South Africa [K]			
	Wood, J.M. 5374 South Africa [K]			
Asclepias aurea	Pegler, A. 954 South Africa [K]		Swaziland's Flora Database: http://www.sntc.org.sz/flora/index.asp	(Nicholas 1982)
	Schlechter, F.R.R. 3409 South Africa [K]			
	Galpin, E.E. 580 South Africa [K]			
	Schlechter, F.R.R. 3409 South Africa [K]			
	Cooper, T. 932 Lesotho [K]			
	Wood, J.M.			

	4555 South Africa [K]			
	Galpin, E.E. 500 South Africa [K]			
Asclepias cucullata	Galpin, E.E. 1034 South Africa [K]		http://www.pbase.com/dorff/image/72262331	(Nicholas 1982)
Asclepias randii	Rand 194 Zimbabwe [K]			(Nicholas 1982)
	Leach L.C & Chase N.C, 10538 Southern Rhodesia [K]			
	Robson & Angus 300 Nyasaland [K]			
Cynanchum ligulatum	Hartweg 1612 Mexico [K]			(Forster 1991)
	Palmer, E. 280 Jalisco [K]			
Pergularia daemia	Peters s.n. Mozambique [K]		Mark Fishbein	(Goyder 2006)
	Kotschy 249 Sudan [K]			
	Drege s.n. South Africa [K]			
Calotropis procera			D. Chuba	(Rahman and Wilcock 1991)
Kanahia laniflora	Millen, H. s.n. Nigeria [K]		kanahia laniflora from www zimbabweflora2 co zw speciesdata species .jpg	(Bullock 1953; Field et al. 1986)
	Bates, G.L. 322		Kanahia laniflora from www	

	Cameroon [K]		zimbabweflora co zw speciesdata family.jpg	
	Staudt A. 603 Cameroon [K] Kumba Johann - Albrechtshöh e		kanahia laniflora from www zimbabweflora co zw speciesdata species .jpg	
	Rowland s.n. Nigeria [K]			
	Speke; Grant s.n. Tanzania [K]			
	Swynnerton, C.F.M. 1895 Mozambique [K]			
	Zenker, G. 1294 Cameroon [K]			
	Swynnerton, C.F.M. 248 Mozambique [K]			
Stenostelma. Capense	Flanagan, H.G. 1693 South Africa [K]			
	Schlechter, F.R.R. 3689 South Africa [K]			
	Lugard, E.J. 82 Botswana [K]			
	Adams, C. 1 South Africa [K]			
	Burchell, W.J. 1791 South Africa			

	[K]			
Asclepias crispa			http://fernkloof.com/species.mv?265	
Aspidoglossum interruptum	Schlechter 11685 South Africa [K]			(Kupicha 1984)
	Schlechter, F.R.R. 3944 South Africa [K]			
	Schimper 1633 Ethiopia [K]			
	Barber, F.W. 847 South Africa [K]			
	Drege 3428 South Africa [K]			
Schizoglossum hamatum	Barber, F.W. s.n. South Africa [K]			(Kupicha 1984)
	MacOwan 1637 South Africa [K]			
	Bowker 11 South Africa [K]			
	Barber, M.E. 29 South Africa [K]			
	Fannin, G. 63 South Africa [K]			
Asclepias syriaca				(Woodson 1954)
Asclepias similis	Ghiesbreght 666 Mexico [K]			(Woodson 1954)
	Coulter 985 Mexico [K]			
	Gómez, R. 809 Guatemala [K]			

	Ghiesbreght 665 Mexico [K]			
Asclepias barjoniifolia	Fiebrig, K. 2663 Bolivia [K]		http://commons.wikimedia.org/wiki/Category:Asclepias_barjoniifolia	(Goyder 2007)
	Bang, M. 982 Bolivia [K]			
	Fiebrig, K. 3088 Bolivia [K]			
	Bridges Bolivia [K]			
Asclepias candida	Claussen, P. Minas Gerais [K]			
Asclepias viridis				(Woodson 1954)
Asclepias stenophylla				(Woodson 1954)
Asclepias oenontheroides				(Woodson 1954)
Asclepias davsii				(Woodson 1939)