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Phylogenetic Placement of African Cornus: Evidence of Nuclear rDNA

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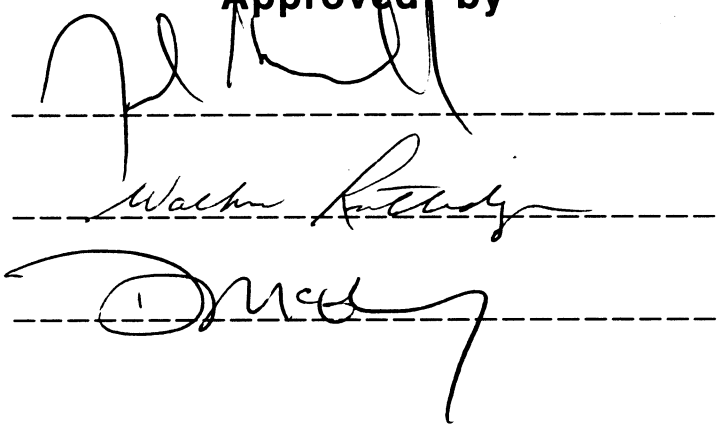
Approved by



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ABSTRACT

The genus *Cornus* (the dogwoods) contains 50 species that have been divided into nine subgenera. One of these species, *C. volkensii*, has been segregated into the subgenus *Afrocrania*. Previous studies combined morphological, anatomical, and chemical data to produce a hypothesis of evolutionary relationships that placed the subg. *Afrocrania* as a segregate lineage sister to the subg. *Cornus*, and placed the *Afrocrania/Cornus* lineage sister to the big-bracted dogwoods (Murrell 1997).

The subgenus *Afrocrania* has been examined morphologically, anatomically, and palynologically; however, it was not sequenced in Xiang's (1993) analysis of *rbc*L (chloroplast) sequence data, or in her analysis of the *matK* region of chloroplast DNA. Murrell's (1993) earlier analysis of the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA included sequence data for *C. volkensii*, but these data were suspect because of inconsistencies with the sequence data for members of the subgenus *Cornus*. Both ITS regions from the subg. *Afrocrania* were amplified using the external primers 4 and 5 and the Polymerase Chain Reaction (PCR), and two distinctly migrating bands (approximately 750 bp in length) were consistently obtained. These amplified regions were cloned in an attempt to isolate these disparate bands for sequencing. Pylogenetic analyses of the sequences revealed that subgenus *Afrocrania* forms an unresolved trichotomy with subgenus*Thelycrania* (the blue- and white-fruited dogwoods) and the Dwarf dogwoods, the cornelian cherries, and the big-bracted dogwood.

INTRODUCTION

The genus *Cornus* (commonly known as dogwoods) comprises 50 species of trees, shrubs, and herbs with centers of diversity in Eastern Asia, North America, and Central America, plus a few species in Africa, Europe, and South America. The genus is broadly defined to include eight subgenera, many of which were at one time treated as distinct genera (Murrell 1997). There is general agreement now that they all represent a single monophyletic group and should be maintained in a single genus (Murrell 1997).

The most recent hypotheses of relationships of *Cornus,* however, are in conflict. Eyde (1988) produced a hypothesis based on morphological characters that placed the dwarf dogwoods (subg. *Arctocrania*) and the big-bracted dogwoods (subg. *Cynoxylon* and *Syncarpea*) sister to one another, with the cornelian cherries (subg. *Cornus* and *Afrocrania*) basal to this derived clade, and the blue-and white-fruited dogwoods (subg. *Thelycrania*) at the base of the evolutionary tree (Fig. 1). Xiang et al. (1993) generated a hypothesis of evolutionary relationships using *rbc*L sequence data that also placed the dwarf dogwoods and big-bracted dogwoods as sister taxa, but the cornelian cherries were basal in the genus (subg. *Afrocrania* was not included). Murrell (1993, 1996) generated a third hypothesis based on morphological, anatomical, biochemical, and cytological data that placed the cornelian cherries sister to the big-bracted dogwoods, with the dwarf dogwoods basal to that clade, the blue-and white-fruited dogwoods (except for subg. *Yinquania*) basal to those lineages, and subg. *Yinquania* placed most basal in the genus (Fig. 2).

Xiang (1995) generated two new hypotheses of relationship, one using the *rbc*L sequence data and a second using both matK and a combined *rbc*L-*mat*K data set. She recognized two major lineages, one with the big-bracted dogwoods and the dwarf dogwoods, and a second clade that includes the blue- and white-fruited dogwoods and the cornelian cherries. Finally, Hardin and Murrell (1997) generated a hypothesis based on foliar micromorphology consisting of a reconstructed cornelian cherry clade supporting an unresolved trichotomy between subg. *Afrocrania*, subg. *Yinquania*, and subg. *Cornus*. Although decay indices for each clade suggested that the support for each one was not strong, the results did support the separation of *Cornus* and *Afrocrania* as separate subgenera (Eriksson and Wikstrom, 1995).

The subgenus *Afrocrania*. is represented by a single species, the African dogwood *Cornus volkensii*, a dioecious plant with a unique pollen type. The purpose of this study was to explore the phylogenetic placement of the African dogwood,*Cornus volkensii*, within the genus *Cornus*, using a molecular analysis of nuclear rDNA.

MATERIALS AND METHODS

Nuclear ribosomal DNA-- Nuclear DNA was chosen for sequencing to avoid problems inherent in comparisons of uniparentally inherited genomes, such as chloroplast DNA. Sequencing the 18S-26S nuclear ribosomal RNA (nrDNA) gene family has proven to be a useful analytical method for phylogeny reconstruction in plants, especially at the family and higher taxonomic levels (Hamby and Zimmer, 1992) as well as among closely related genera or species (e.g., Sytsma and Schaal, 1985; Rieseberg, Soltis, and Palmer, 1988; Kim and Mabry, 1991). The nuclear ribosomal DNA gene family codes for the three subunits which comprise the ribosome of eukaryotes. These sequences are tandemly repeated within the genome, and a single genome may contain as many as 14,000 copies (Long and Dawid, 1980). The ribosomal gene family is transcribed as a single unit with the sequences for all three ribosomal subunits carried by a single strand of mRNA. Three noncoding sequences are transcribed along with the coding sequences. Two of these non-coding sequences separate the genes coding for the ribosomal subunits and are referred to as the internally transcribed spacers (ITS). The first region, ITS 1, separates the 18S gene from the 5.8S gene, and the second region, ITS 2, separates the 5.8S gene from the 26S gene. The third transcribed noncoding region is downstream from the coding regions and is therefore referred to as the externally transcribed spacer. An untranscribed intergenic spacer (IGS) separates the transcribed regions (Campbell *et al.* 1995).

As the ITS regions are noncoding, they may undergo random mutation without the evolutionary pressure for sequence conservation to which coding regions are subjected. The sequence of the ITS regions may therefore diverge in separate lineages, providing information as to the evolutionary history of a given taxon. Since the ITS regions are flanked by the highly conserved genes coding for the ribosomal subunits, primers can be designed which will allow for the rapid replication of the sequence via the polymerase chain reaction (PCR). The replicated ITS units may then be sequenced by the dideoxy sequencing method (Sanger *et al.* 1977).

The ITS sequence data has been used in vascular plants to describe species divergence within a single genus (Wojciechowski *et al.* 1993; Hsiao *et al.* 1994; Nickrent *et al.* 1994), as well as generic relationships within a family (Baldwin 1992; Suh *et al.* 1993; Campbell *et al.* 1995). The basis of these studies rests upon the assumption that ITS sequence is conserved within species. The ubiquity of rRNA genes in nature and considerable evidence that the repeated unit consists of regions that have different rates of sequence divergence account for its phylogenetic utility. No published data was found regarding ITS sequence divergence in vascular plants below the species level, although work by Murrell (pers. comm.) suggests that ITS sequences may be used to distinguish hybrids between *Cornus obliqua* Raf. and *C. amomum* Miller. If ITS sequence data could be used to delineate intraspecies divergence, the distance estimates among the populations could be based on fully comparable data. External primers 4 and 5 were used to amplify ITS regions I and II (Fig. 3).

Plant samples-- Extracted DNA was received from Dr. John Knox, who obtained terminal first-year portions of stems containing 5 to 10 leaves, and who removed a single individual to provide genetic vouchers for DNA sequencing. This tissue voucher was placed in a ziplock bag and was quickly stored at 4°C in a cooler containing ice. The sample was then processed in the lab. The tissue was transferred to a clean ziplock bag marked with the control number and stored in a -80 °C freezer until DNA extraction.

Whole genomic DNA was extracted from the leaves following the CTAB extraction procedure of Doyle and Doyle (1987). Fifty mg of leaf tissue were rehydrated in distilled water for 15 minutes. The leaf tissue was then soaked in liquid nitrogen for 20 minutes and ground in a pre-cooled (-80°C) mortar. The grindate was transferred to1.5 ml microfuge tubes containing 0.8 ml of CTAB buffer [2x CTAB, 1% PVP, 1% Sodium bisulfate] and 0.2 % ß-Mercaptoethanol that had been heated at 60°C for 15 minutes. The tubes were capped, inverted several times, and heated at 60°C for 30 minutes with occasional inverting. Five hundred fifty µl of 24:1 chloroform:isoamyl alcohol solution were added to each tube and inverted. The tubes were spun at top speed in a microcentrifuge for 5 minutes. The aqueous layer was removed and placed in a clean microfuge tube. Added to each tube were 0.4 ml of ice-cold isopropanol. The tubes were mixed by inversion. The tubes were incubated overnight at -20°C to allow the DNA to precipitate. The tubes were spun at top speed for 10 minutes in a microcentrifuge to pellet the precipitated DNA. The supernatant was discarded, and the pellet was dissolved in 0.2 ml of TE buffer at 37°C. To each tube, 0.1 ml of 7.5 M ammonium acetate and 0.6 ml of 100% ethanol were added. The DNA was allowed to precipitate at -20°C for 10 minutes, and the tubes were spun in a microcentrifuge for 5 minutes at full speed. The supernatant was discarded, and the pellet was dissolved in 0.2 ml of TE buffer at 37°C. Added to each tube were 20 µl of 2.5 M sodium acetate and 0.44 ml of 100% ethanol, and the tubes were inverted to mix. The DNA was allowed to precipitate at -20°C for 10 minutes, and the tubes were spun in a microcentrifuge for 5 minutes at full speed. The supernatant was discarded, and the pellet was covered with 70% ethanol for 5 minutes. The ethanol was discarded, and the pellet was allowed to air dry. Residual alcohol was removed by drying in a speedvac for 5-10 minutes. The pellet was resuspended in 0.1 ml of TE buffer at 37°C for 30 minutes. The DNA samples were stored at -20°C. The stock DNA extraction solutions were diluted (1:100) to yield DNA concentration of 1.0 to 100 ng.

Polymerase chain reaction-- Polymerase chain reaction (Wojciechowski *et al.* 1993) mixtures totaling 25.0 µl were prepared. These reaction mixtures contained 12.5 µl diluted genomic DNA, 2.9 µl molecular grade water, 2.5 µl 50% glycerol, 2.5 µl 10x PCR buffer, 1 µl of Taq polymerase, 2.0 µl of a dNTP mixture (0.5 µl each of dATP, dGTP, dCTP, and dTTP), and 1.25 µl of both ITS primer 4 and ITS primer 5 (White *et al.* 1990) in equimolar concentrations. The amplified DNA was further purified using a Gene Clean II kit. After running the DNA on an agarose gel, the DNA bands were excised from the gel and transferred to microfuge tubes. Three volumes of NaI were added, and the mixtures were heated to 55°C. The tubes were inverted to mix, and 5 µl of glassmilk® suspension were added to each tube. The suspensions were pelleted in a microcentrifuge, and the NaI supernatants were decanted. The pellets were washed with 700 µl of ice cold New Wash solution®, and the pellets were allowed to resuspend. The solutions were pelleted again in a microcentrifuge, and the DNA pellets were resuspended in TE buffer (Geneclean II Kit, Bio 101 Inc.). Presence of DNA was determined by agarose gel electrophoresis of DNA stained with 0.5 µg/µl ethidium bromide to allow for visualization under UV light.

Cloning-- Products of the PCR reactions that ran on the electrophoresis gel at disparate rates were separated via cell transformation and cloning procedures according to Invitrogen's Original TA Cloning Kit™ (Catalog no. K2000-01). PCR products less than 1 day old were ligated to pCR 2.1, a 3.9 kb plasmid, in an overnight ligation reaction at 14°C. The recombinant plasmid was then used to transform INValphaF' competent cells. The cells were thawed on ice, and 2 µl ß-Mercaptoethanol were added to disrupt cellular protein disulfide bonds. Two µl of the ligation reactions were added to the cells, and the mixtures were incubated at 4°C for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C and then placed on ice for 2 minutes. Two hundred and fifty µl of SOC broth was then added, and each tube was placed in a shaker at 37°C for 60 minutes at 225 RPM. The tubes were placed on ice. Fifty µl of the reaction mixtures were then spread on separate agar plates of LB + 60 µg/ml ampicillin containing 40 µl of 40 mg/ml X-Gal. The liquid was allowed to absorb into the agar, and the plates were inverted and placed

into a 37°C incubator for 21 hours. The plates were then incubated at 4°C for 2 hours for color development.

Small scale plasmid preparation-- Four white colonies were picked from each transformation-reaction plate and grown overnight in 3 ml LB + 50 μg/ml ampicillin. Cells were pelleted by centrifugation for 2 minutes, and the pellets were resuspended in 200 μl cell resuspension solution, according to Invitrogen's Wizard Miniprep Kit™. Two hundred μl of cell lysis solution were added and mixed gently with the cells. Finally, 200 μl of neutralization solution were added and mixed with the cells. The reactions were spun in a microcentrifuge at maximum speed for 5 minutes. The supernatant of each reaction was removed and placed into new tubes. One ml of purification resin was added at 29 °C to the supernatant and mixed by inversion. The mixtures were gently pushed through a mini-column filter attached to a syringe, and 2 ml of column wash solution were added to the syringe and pushed through the mini-column as well. The mini-columns were transferred to fresh microcentrifuge tubes and spun at maximum speed for 20 seconds to dry the resin. The mini-columns were, again, transferred to fresh microcentrifuge tubes, and 50 μl of nanopure, double de-ionized, autoclaved water (at 55 °C) were added to the mini-columns. The tubes were spun at top speed for 20 seconds to remove the DNA and water from the mini-columns. The plasmid DNA was stored at -20°C.

Restriction digestion analysis-- The isolated plasmids were analyzed by restriction digestion to confirm the ligation and separation of PCR products. Ten µl of each isolated plasmid pCR 2.1 + PCR insert were added to 20 µl reactions containing 1.8 µl Hind III (@ 10 U/µl), 2.0 µl 10x React II buffer (Gibco BRL Enzymes®), and 6.2 µl autoclaved ddH_2O. The reactions were incubated at 37°C for 6 hours and were visualized on a 1% agarose gel stained with 0.5 µg/ml ethidium bromide.

DNA sequencing-- Cycle sequencing of DNA was performed using the dideoxy sequencing method of Sanger *et al.* (1977). The primer used was end-labelled with [gamma-^{33}P] ATP by adding 1.0 pmol of primer (ITS primers 2, 3, 4 and 5) to 1 µl 5x kinase buffer, 0.5 µl ^{33}P ATP, and 1.0 µl T4 kinase. This solution was diluted with water to yield a final volume of 5.0 µl. The reaction was incubated at 37°C for ten minutes, followed by incubation at 55°C for five minutes. A pre-reaction mixture was made containing 5.0 µl of the labelled primer, 4.5 µl 10x Taq sequencing buffer, 2 µl DNA, 24.0 µl molecular grade water, and 0.5 µl Taq DNA polymerase.

Four termination mixtures were subsequently prepared by adding 8.0 µl of the pre-reaction mix to 2.0 µl of dNTP containing dideoxy nucleotides adenine, cytosine, guanine, or thymine. The tubes were placed in a preheated 95°C thermocycler for incubation at 95°C for three minutes. The sequencing process consisted of 20 cycles with a 30-second denaturing step at 95°C, a 30-second annealing step at 55°C, and a 60-second extension/termination step at 70°C. At the end of twenty cycles, 5.0 µl of stop solution were added to each reaction tube. Each tube was then heated at 90°C for 5 minutes.

Sequences were resolved by electrophoresis through 5% acrylamide-8 M urea gels that were run on Owl buffer-backed vertical gel rigs at 100 watts for 2.5 hours for the "short gels" and 5 hours for the "long gels." The gels were fixed by a 30-minute soak in a solution of 5% methanol/5% glacial acetic acid. The gels were then transferred to blotting paper and were vacuum dried at 40°C for 2 hours.

Autoradiographs were produced through the exposure of BioMax MR2 Autorad film for a period of 24 to 72 hours, depending on the activity of the ^{33}P. Films were developed, and the sequences were read manually. The sequences were stored in SeqEd v1.03 to aid in comparison of the nucleic acid sequences. Sequences were aligned with other dogwood data sets using the MacIntosh program CLUSTAL and by manual alignment.

The MacIntosh program PAUP was used to generate phylogenetic trees. Trees were generated using the heuristic method with subg. *Yinquania* as the outgroup. The results were analyzed for robustness using bootstrap analysis and decay analysis.

RESULTS

The PCR product of the ITS region yielded two distinct bands (~750 bp) running at different rates upon gel electrophoresis. The PCR products were ligated into pCR 2.1, and this vector was used to transform INValphaF' competent cells. Upon plating, over 80% of the colonies were white, indicating a successful ligation of insert within the plasmid. Of the four colonies picked for small-scale plasmid preparation and restriction-digestion analysis, clones A and C exhibited identical bands of ~4600 bp upon gel electrophoresis, and clones B and D exhibited identical bands running at both ~4600 bp and ~4700 bp. Clones A and B were selected for sequencing.

The complete ITS I and ITS II sequences for clone A were alignable with the other dogwood sequences. The sequences of ITS regions I and II for clone B, however, were unalignable with the other dogwood sequences. ITS I sequence (for clone A) was 251 bases long, including 11 bases of the 18S region and 8 bases of the 5.8S region. ITS II sequence was 340 bases long, including 48 bases of the 5.8S region and 11 bases of the 26S region.

The analysis generated three trees of equal length. This variation was in the placement of subg. *Afrocrania*, either basal to the subg. *Thelycrania* and the big-bracted/cornelian cherry/dwarf dogwood clade or sister to the big-bracted/cornelian cherry/dwarf dogwood clade (with subg. *Thelycrania* basal to this clade). The consensus tree represented the three clades (subg. *Thelycrania* clade, the big-bracted/cornelian cherry/dwarf dogwood clade, and subg. *Afrocrania*

clade) as an unresolved polytomy. The consensus tree had a length of 572 steps, a consistency index (CI) of 0.751, and a CI of 0.636 when the uninformative characters were excluded. The bootstrap analysis using a 50% majority bootstrap consensus tree showed support at 64% for the unresolved polytomy of subg. *Thelycrania*, *Afrocrania*, big-bracted dogwoods, dwarf dogwoods, and cornelian cherries. Decay indices (DI) showed the big-bracted dogwood clade with the strongest support (DI = 8), the big-bracted/cornelian cherry clade had a DI of one, the big-bracted/cornelian cherry/dwarf dogwood clade had a DI of one. The unresolved trichotomy of subg. *Thelycrania*, subg. *Afrocrania*, and the big-bracted/cornelian cherry/dwarf dogwood clade had a DI of three.

Using these results, a hypothesis of relationships was made, with subgenus *Yinguania* as the outgroup, section *Umbellicrania* is next most basal, and subg.*Afrocrania* is sister to the rest of the genus (Fig. 4). Subgenus *Afrocrania* forms an unresolved trichotomy with subg.*Thelycrania* (the blue- and white-fruited dogwoods) and the Dwarf dogwoods, the cornelian cherries, and the big-bracted dogwoods (Fig. 5).

CONCLUSIONS

The ITS hypothesis of relationships, if correct, suggests that cavities in the seed stone and the umbel-like inflorescence evolved twice in the genus. These results suggest that the direction of evolution from the basal lineage resulted in two distinct lineages (Fig. 6), one with bracts extended along the entire length of the primary branch (subg.*Yinquania*), and another with bracts adnate to only a portion of the primary inflorescence branch (subg. *Thelycrania*). If the partial bract-slide inflorescence is ancestral to the rest of the genus, then it would necessitate a primary branch collapse in the subg. *Afrocrania* lineage and in the cornelian cherry/big-bracted lineage (Fig. 7). The molecular results are in conflict with hypotheses derived from morphological (Murrell 1993) and micromorphological data (Hardin and Murrell 1997). If molecular data may be considered as more informative, this conflict suggests that more work is needed to reanalyze morphological structure to resolve these conflicts.

Two additional aspects of these results provide new avenues for further investigations. First, subg. *Umbellicrania* and subg. *Afrocrania* were shown to be basal lineages in the genus, and these two groups are only found in the southern hemisphere. Because the genus *Cornus* has the highest levels of diversity in Eastern Asia (twenty out of fifty species), Asia has generally been considered the region of origin for the genus. The results of the molecular analyses are in conflict with this hypothesis, because these two basal lineages do not have an Asian distribution. Secondly, all three basal lineages (subg. *Yinquania, Afrocrania,* and *Umbellicrania*) exhibit a tree architecture. The remainder of the genus are shrubs or herbs with woody rhizomes, excluding subg. *Discocrania*. The ancestor to the dogwoods, then, may have possessed a treearchitecture.

These data and their accompanying conclusions are complicated by the possible problem of long-branch attraction (Hillis et *al.* 1994). Long-branch attraction occurs when two DNA sequences converge and seem to suggest evolutionary relatedness when no relatedness truly exists. The sequences for subg. *Afrocrania* were somewhat divergent from the rest of the genus, and

therefore difficult to align. A different segment of the genome may need to be sequenced and compared in order to elucidate genuine evolutionary relationships.

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FIGURES

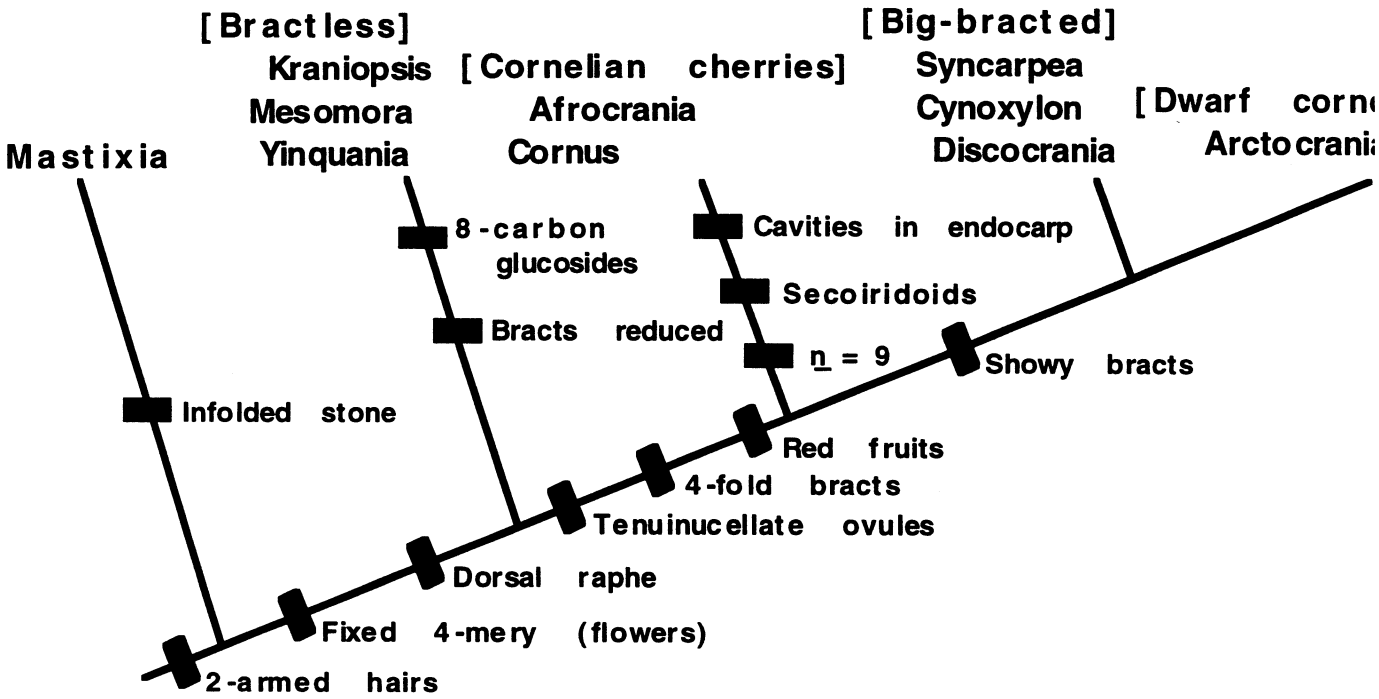


Figure 1. Eyde's (1988) hypothesis of relationships of the dogwoods, based upon morphology and biochemistry.

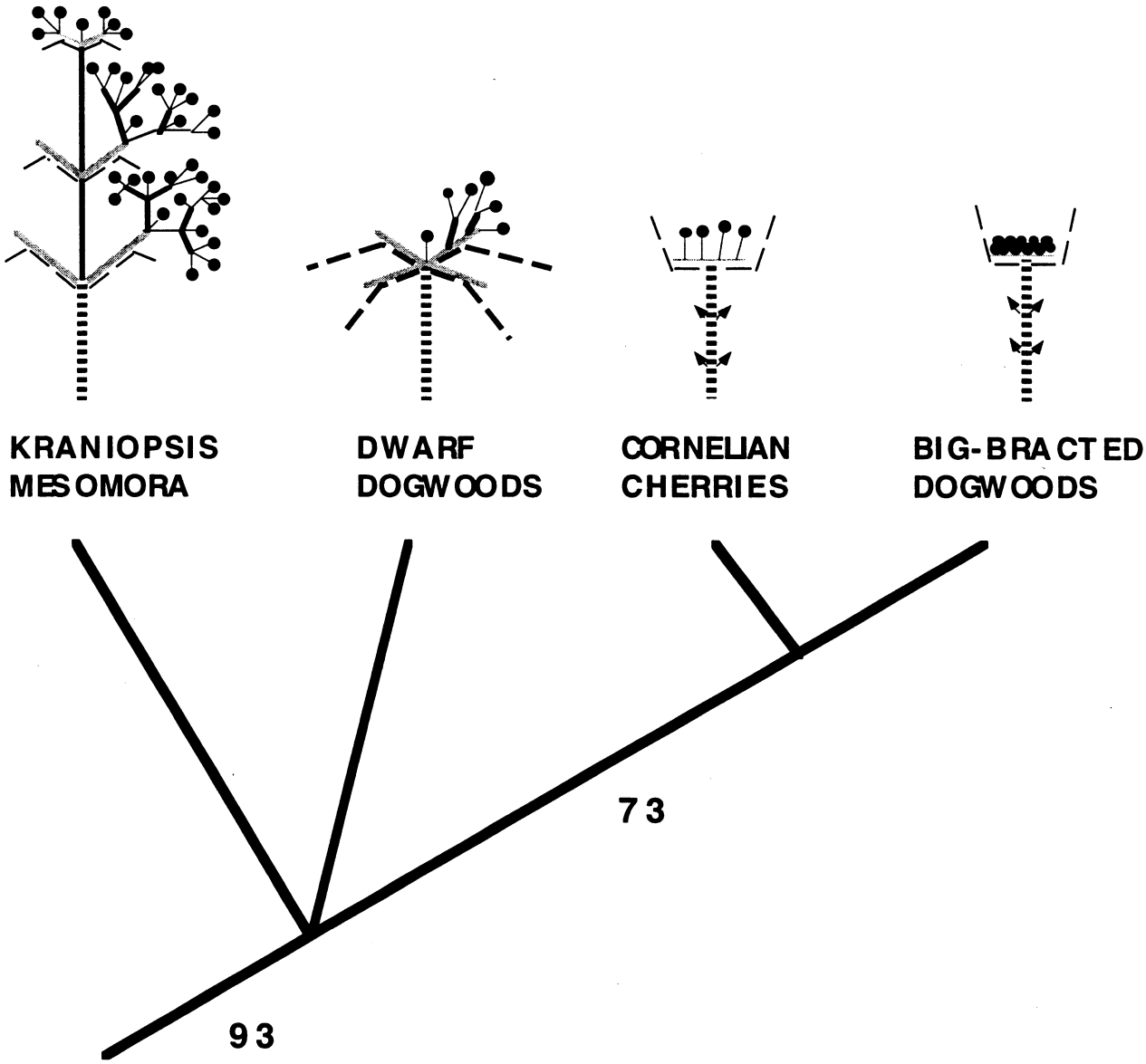
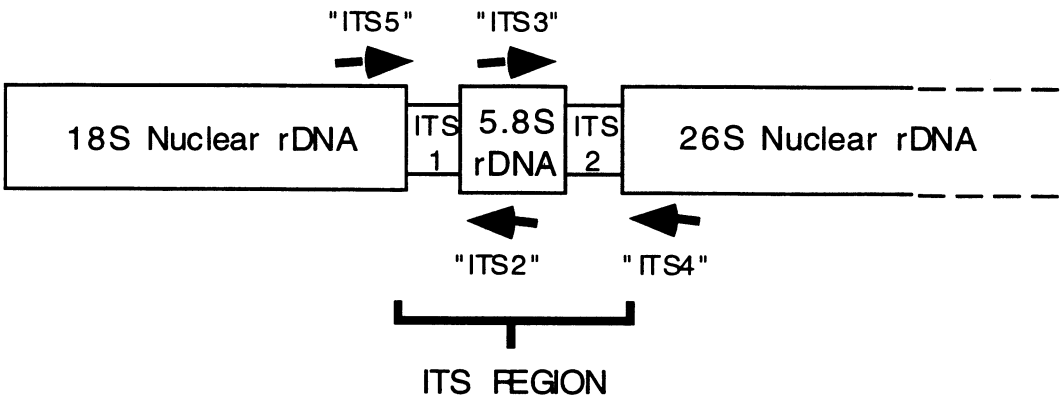


Figure 2. Murrell's (1993, 1996) hypothesis of relationships in the dogwoods. Murrell included subg. *Afrocrania* within his concept of the Cornelian Cherries in the 1993 analysis, and subg. *Afrocrania* was not included in the 1996 analysis. Numbers at the nodes represent the bootstrap values (an indication of support) for the clades.

ITS Region = Internal Transcribed Spacer Region



Repeat unit of 18-26S nuclear ribosomal DNA, minus the intergenic spacer and much of the 26S subunit. Arrows indicate approximate positions of primers used to amplify single or double-stranded DNA for sequencing.

Figure 3. Schematic of the Internal Transcribed Spacer Region. ITS primers 4 and 5 were used to amplify the region.

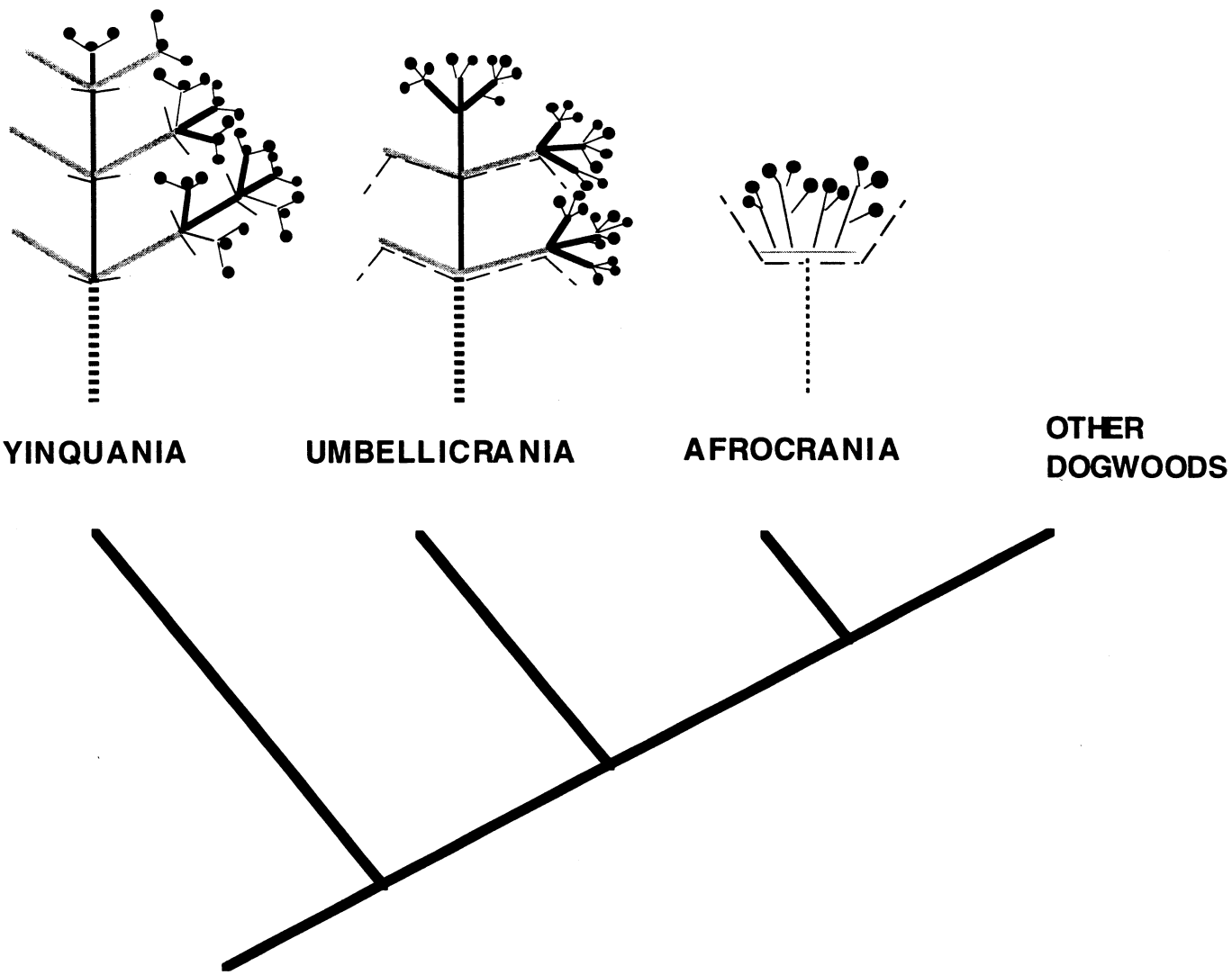


Figure 4. A hypothesis of relationships based upon the total ITS data. Subgenus *Yinguania* is the outgroup, section *Umbellicrania* is the next most basal, and subg.*Afrocrania* is sister to the rest of the genus.

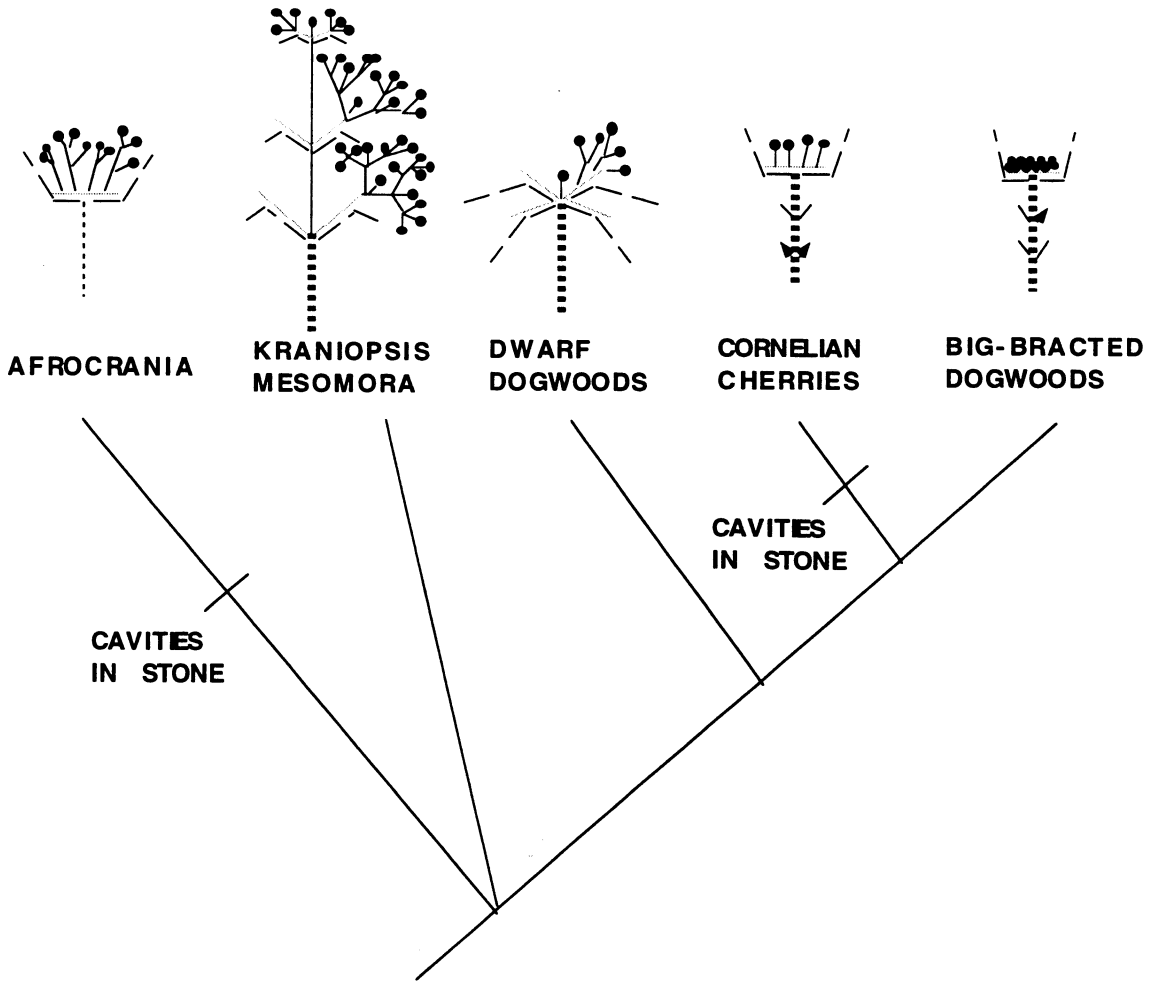


Figure 5. A hypothesis of relationships based upon total ITS data. Subgenus *Afrocrania* forms an unresolved trichotomy with subg. *Thelycrania* (the blue- and white-fruited dogwoods) and the Dwarf dogwoods, the cornelian cherries, and the big-bracted dogwoods. Cavities in the seed stone and the umbel-like inflorescence seem to have evolved twice in the genus.

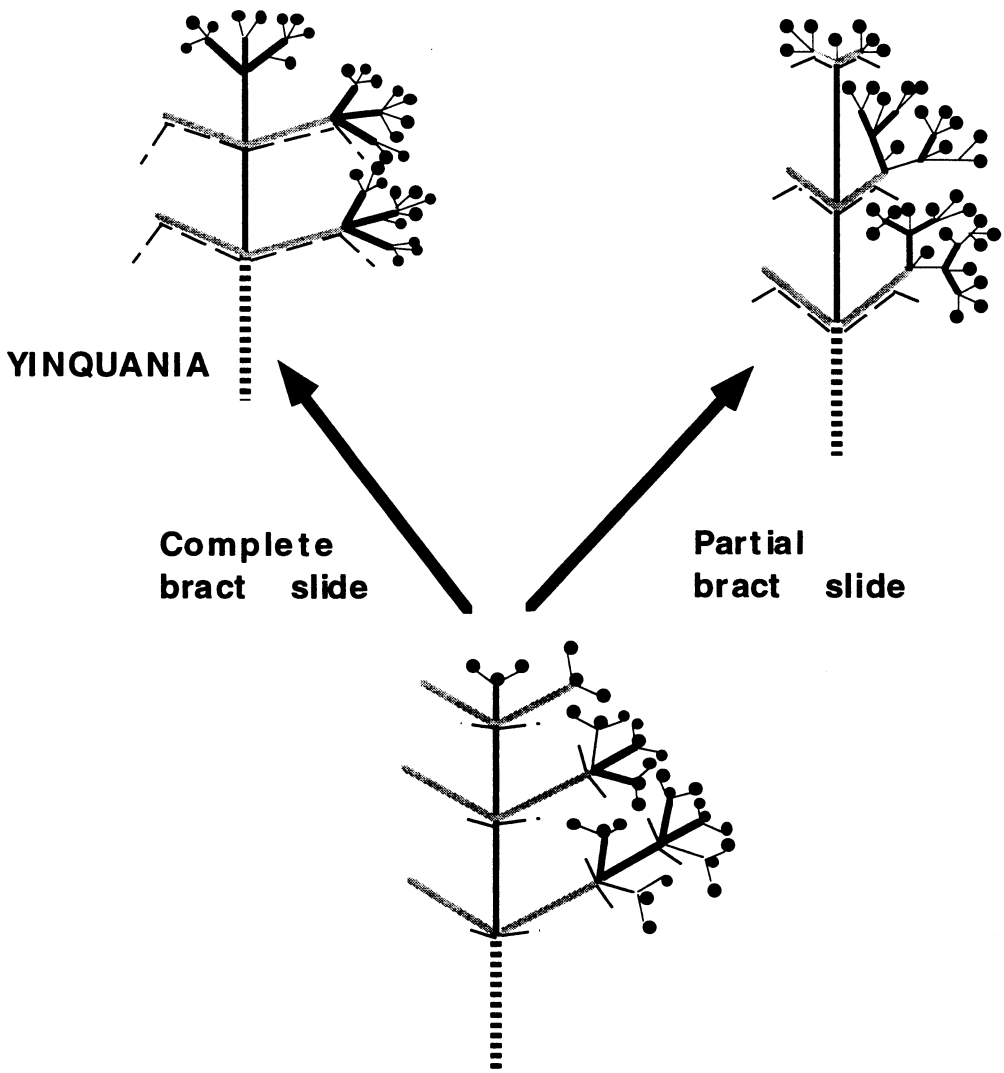


Figure 6. The direction of evolution from the basal lineage resulted in two distinct lineages, one with bracts extended along the entire length of the primary branch (subg. *Yinquania*), and another with bracts adnate to only a portion of the primary inflorescence branch.

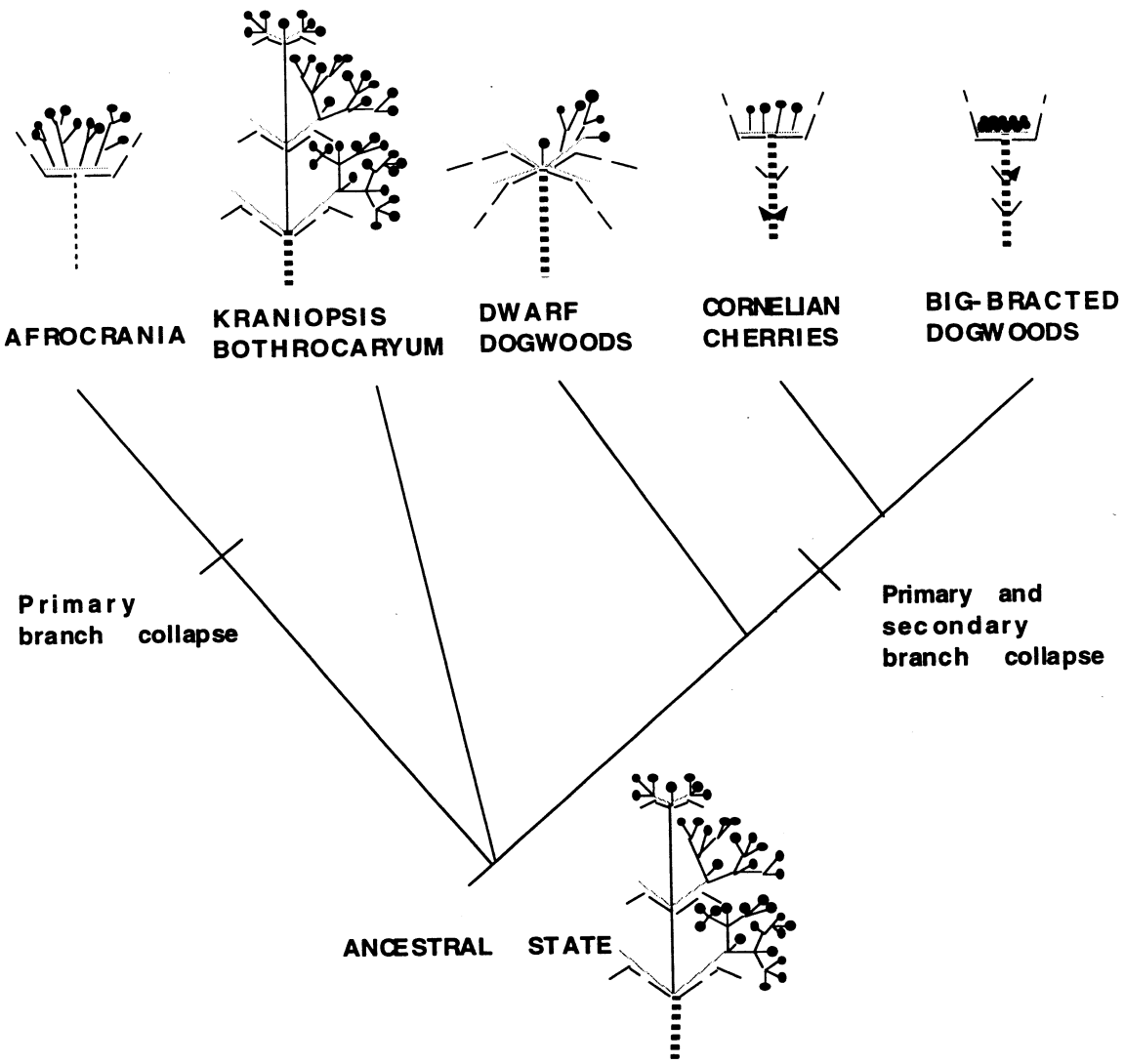


Figure 7. A hypothesis of relationships. If the partial bract-slide inflorescence is ancestral to the rest of the genus, then it would necessitate a primary branch collapse in the subg. *Afrocrania* lineage and in the cornelian cherry/big-bracted lineage.

APPENDIX

Raw sequence data from clone A. The 5-2 sequence is from ITS I and the 3b-4 sequence is from ITS II.

Afrocrania ITS I

GaacgcagaatcggcttgaagaaaagcgtaacaaggtttcctgaggtgaacctcgGAAG
GAtCATTACCTAATATAtCAnATGGGGGGtCGCCAGCAATACCCTATA
GCGAGGTGAAAACGTGATTTAGTGTGTATCAGACCCCTCTATTGACT
ATACCCATGTCTTTTGCGTACGATTTGTTTCCTTGGTAGGcTTGCCTGC
CAAT?GGaCATTTATACAACCCTTTGTAATTGCAGTCAGCGTcAgAA
AACAAACAATAATTACAACttttaacaacggatctcttggttctggcatcgatga
agaacgcgcgaatg

Afrocrania ITS II

AkAAgTagTGyaGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
ArCGCACATTGCGCCCCTTGGTATTCCAGGGGgCATGCCTGTTCGAgCGt
CATTTGTACCCTCAAGCTCTgCTTGGTGTTGGGTGTTTGTCTCGCCTTGG
TGCGCAGACTCGCCkkAAwACAATTGGCAGCCGGCAT?TTAGCTTGGA
GCGCAGcACATTTTGCGTCCCTTGATAACTGTTGTTGGmACCCATtCA
AGThcaTTTATTTGCTCTTGACCTCGGwTCAGgta