Mitochondrial DNA Variation in the
Dionda episcopa species complex

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INTRODUCTION

Until recently, the cyprinid fish genus *Dionda* contained eight species: *D. catostomops, D. couchi, D. diaboli, D. dichroma, D. episcopa, D. erimyzonops, D. ipni,* and *D. manibularis* (Hubbs and Brown, 1956; Hubbs and Miller, 1977). Most species of this genus are found in river drainages ranging from eastern New Mexico to western Texas, south into north central Mexico and over into eastern Mexico. Only two species of *Dionda*, *D. diaboli* and *D. episcopa*, are found in the continental United States. *Dionda episcopa* is thought to be a complex of several different forms (Hubbs and Miller, 1977). In a recent phylogenetic study of allozyme variation, Mayden et al. (1991) split the *Dionda episcopa* complex into five species. These included: *D. argentosa* and *D. diaboli* in the Devils River and San Felipe Creek, *D. episcopa* in the Pecos River, *D. serena* in the Nueces River, and *D.* sp., an undescribed species in the Colorado/Guadelupe River drainage. The phylogenetic hypothesis proposed by Mayden et al. (1991) for these species is shown in Figure 1.

The purpose of this study was to test the phylogenetic hypothesis proposed by Mayden et al. (1991) using restriction fragments of mitochondrial DNA (mtDNA). MtDNA provides another method by which a phylogenetic hypothesis can be inferred. The advantages of using mtDNA in phylogenetic analysis are severalfold. The molecule is small, physically circular, and transmitted maternally (Brown, 1983). In addition, the mutation rate in animal mtDNA is estimated to be from 5 to 10 times faster that that of single copy nuclear DNA (Brown, 1985). Because of the above, mtDNA has

proven extremely useful in phylogenetic analysis, especially when dealing with closely related species (Bermingham and Avise, 1986; Moritz et al., 1987; Avise et al., 1987; Dowling and Brown, 1989).

MATERIALS AND METHODS

Specimens were collected by seine, frozen immediately in liquid nitrogen, and stored at -80°C. Sample localities (number of specimens) are as follows: *Dionda argentosa* -- Devils River at Bakers crossing, Hwy 163, Val Verde Co., TX (5), and Devils River at the Texas Parks and Wildlife Department refuge, Val Verde Co., TX (7); *D. diaboli* -- San Felipe Creek at Del Rio, Val Verde Co., TX (7); *D. episcopa* -- Cottonwood Creek, Eddy Co., NM (6), and Bitter Creek, Chaves Co., NM (6); *D. serena* -- Nueces River at Hwy 55, Real Co., TX (7), Frio River at Concan, Uvalde Co., TX (5), and Sabinal River at Lost Maples, Bandera Co., TX (6); *D.* sp. -- Fesenden Spring, Kerr Co., TX (7), and Concho River at Anson Springs, Tom Green Co., TX (7). The outgroup taxon used in the study was *Hybognathus nuchalis*. Specimens of *H. nuchalis* were obtained from the Ouachita River in Bradley Co., AR (6). A map of the sampling localities of the *Dionda* species is shown in Figure 2.

Entire fish were pulverized into a fine powder in liquid nitrogen using a prechilled mortar and pestle. About 1g of powder was suspended in STE buffer (pH 7.5). Cells were then lysed by adding 20% sodium dodecyl sulfate in water (0.5 mg/ml). This mixture was shaken vigorously and immediately extracted twice

with an equal volume of a 25:24:1 solution of phenol:chloroform:isoamyl alcohol. Samples were centrifuged for 4 min at 14,000 rpm during each extraction to facilitate separation of the layers. The supernatant was then extracted twice with a 24:1 solution of chloroform:isoamyl alcohol, and centrifuged as before. DNA was precipitated by the addition of a 1/10 volume of 2.5M sodium acetate (pH 5.2) and two volumes of absolute ethanol and placed at -20°C for two hours. Precipitated DNA was then recovered by centrifuging at 8,000 rpm for 10 min at 4°C. The DNA was then dissolved in a solution of 100 mM Tris, 10 mM EDTA (pH 7.5).

Ten hexameric restriction endonucleases (*Bg*l II, *Bst*E II, *Nco* I, *Nhe* I, *Nsi E*, *Pst* I, *Pvu* II, *Sca* I, *Sst* I, and *Sst* II) were used to digest 1.0 to 1.5 µg of DNA in 40 µl reactions following the enzyme manufacturer's specifications. DNA fragments were then electrophoresed at approximately 5V/cm for 15 hr in 0.8% agarose gels (buffer system: 0.8 M Tris, 0.4 M sodium acetate, 0.36 M NaCl, 0.04 M EDTA; pH 8.0). Lambda DNA cut with the restriction enzyme *Hin*d III was used as the molecular weight standard on each gel. After electrophoresis, the DNA was stained with ethidium bromide, viewed, and photographed under 300 nm UV light. The gels were denatured in 0.5 N NaOH for 1 hr, and the DNA fragments transferred to nylon filters (Micron Separations Inc. 0.45 micron) following the method of Southern (1975). Southern blots were then hybridized to a probe labelled with [^{32}P]dCTP via random priming (Feinberg and Bolgelstein, 1984). The probe used was the entire mtDNA molecule of the cyprinid, *Cyprinella lutrensis*, cloned into lambda bacteriophage using EMBL arms. After hybridization, filters were

washed and exposed to X-ray film with intensifying screens for 36 to 70 hr at -80°C in order to visualize the mtDNA fragments.

Restriction fragment presence/absence (binary) matrices for parsimony and phenetic analyses were constructed using individual mtDNA haplotypes and individual sampling localities (= samples) as operational taxonomic units (OTUs). The presence or absence of a fragment was considered a character state (cs). For the samples, a restriction fragment was scored as present (cs=1) if it occurred in an individual in the sample, or absent (cs=0) if it did not.

Parsimony analysis of the fragment presence/absence matrix of mtDNA haplotypes was used to produce a "gene" tree (*sensu* Avise, 1989) using the MULPARS option in version 3.0 of the Phylogenetic Analysis Using Parsimony (PAUP) program of Swofford (1985) and the Boot program in version 3.0 of the Phylogeny Inference Package (Phylip) of Felsenstein (1987). The trees were rooted using *Hybognathus nuchalis* as the outgroup. All autapomorphies (52 cs) and symplesiomorphies (1 cs) were removed from the haplotype binary matrix, leaving 152 phylogenetically informative characters. The same analysis was performed on the sample fragment presence/absence matrix. All autapomorphies (96 cs) and symplesiomorphies (1 cs) were removed from the haplotype binary matrix, leaving 108 phylogenetically informative characters.

Nucleic sequence divergence matrices were calculated using the haplotype and sample fragment presence/absence matrices using equations derived by Nei and Li (1979). These matrices were then used to construct a phenogram using UPGMA clustering (Sneath and Sokal, 1973).

RESULTS

Single digestions of mtDNAs from the 62 individuals examined (including 6 individuals of *H. nuchalis*) using the ten restriction enzymes resulted in 205 unique fragments. In most cases, homology of individual fragments was tested with side-by-side comparisons of the variant patterns produced by each enzyme. Homology was assumed if fragments were the same size in side-by-side comparisons. An example of variant single-digestion patterns for the enzyme *Bgl* II are shown in Figure 3. Digestion patterns of all 10 restriction enzymes were used to generate composite digestion patterns. Twenty-one unique composite patterns or haplotypes were found among the 56 *Dionda* examined and three haplotypes were found in the *H. nuchalis* examined.

Parsimony analysis (using PAUP) and phenetic clustering (using UPGMA) of the clonal fragment presence/absence and percent nucleotide sequence divergence matrices, were carried out to determine if there were any rare haplotypes which might compromise phylogenetic analysis. If all clonal types from a single sample occur in a monophyletic cluster, then all haplotypes can be included in analyses of samples. No outlyers were found. Cladistic and phenetic analyses were then performed on the sample fragment and on the percent nucleotide sequence divergence matrices (OTU=sample).

Parsimony analysis of the sample fragment presence/absence matrix yielded one minimum length tree (Figure 4). The bootstrapping values obtained using Phylip strongly supported the

intraspecies pairs obtained. Ninety-six percent of replicates support monophyly of the two samples of *D. argentosa*, one hundred percent of replicates support monophyly of the two samples of *D. episcopa*, and eighty-four percent of replicates support monophyly of the Frio River and Sabinal River samples of *D. serena*. Monophyly of *D. episcopa* and of the Nueces River sample of *D. serena* was supported by ninety-six percent of replicates. All other nodes were not supported in more than fifty percent of replicates.

The UPGMA-generated phenogram of similarity among samples is shown in Figure 5. The groupings of samples using phenetic analysis are similar to those inferred from parsimony analysis. The only nodes in the UPGMA analysis that are under five percent sequence divergence are between samples of the same putative species which differ by less than one percent. Not all "species" were distributed in monophyletic assemblages. These included *D.* sp. and *D. serena*. The sample nucleotide divergences between samples of these two "species" differed by well over five percent. Considering the low intraspecific sequence divergences reported within species of other North American minnows (i.e. less than or equal to one percent) (Richardson and Gold, 1990), this may suggest the presence of different species with *D.* sp. and *D. serena*.

DISCUSSION

The data from this study suggest that *D. serena* and *D.* sp. as presently constituted are not monophyletic. However, excluding the placements of *D. diaboli*, the Concho River *D.* sp., and the Nueces River *D. serena*, the phylogeny inferred from mtDNA is concordant with the phylogeny inferred by Mayden et al. (1991).

The phenogram derived from mtDNA data is identical to the phylogenetic tree. Samples of the same species that occur as sister groups on the phylogenetic tree also cluster on the phenogram. The estimated sequence divergence for these sister groups are less than 1%. The estimated percent sequence divergence for all other nodes ranged from 6.69% to 14.63%.

The divergence of the Nueces River sample of *D. serena* from other *D. serena* suggests the former may represent a distinct species. Because mtDNA has higher resolving power than allozymes, the occurrence of a distinct species in the Nueces River may not have been distinguishable with the allozyme data of Mayden et al. (1991). Each sample of *D. serena* was collected from the headwaters of the Nueces, Sabinal, and Frio Rivers. This geographic distance may have been adequate to allow the Nueces River population to diverge significantly from the Sabinal and Frio River populations. A similar biogeographic pattern occurs in *Cyprinella leprida* (L. Richardson, pers. comm.).

D. sp., as described by Mayden et. al. (1991), occurs in two drainage systems, the Guadelupe River drainage and the Colorado River drainage. When doing the allozyme study of the *Dionda*

episcopa complex, Mayden et. al. (1991) only sampled one population from each drainage. Mayden et al.'s Fesenden Spring sample represented the Guadelupe River drainage, and a sample from Bailey Creek represented the Colorado River drainage. In this study, single collections also were taken from each drainage system. The Fesenden Springs sample of *D.* sp. represented the Guadelupe River drainage, and the Concho River sample represented the Colorado River drainage in this study. Our sample from the Guadelupe River drainage is placed exactly where Mayden et. al.'s *D.* sp. (from the same locality) was in their phylogeny (Figures 1 and 4). Our sample from the Colorado drainage system (i.e. the Concho River sample) falls as sister to *D. argentosa,* far removed from the placement of the Fesenden Springs sample of *D.* sp. on the phylogenetic tree (Figure 4). Mayden et al.'s sample and the sample for this study representing the Colorado River drainage are probably geographically isolated. Perhaps if we had sampled the same population from Bailey Creek, our results would have concurred with those of Mayden et al. (1991). The Concho River locality is found north of the Bailey Creek locality. Given that *Dionda* prefer clear running water, the main body of the Colorado River could act as a barrier to gene flow. For this reason, it is possible that the *D.* sp. from the Concho River represents another species of *Dionda.*

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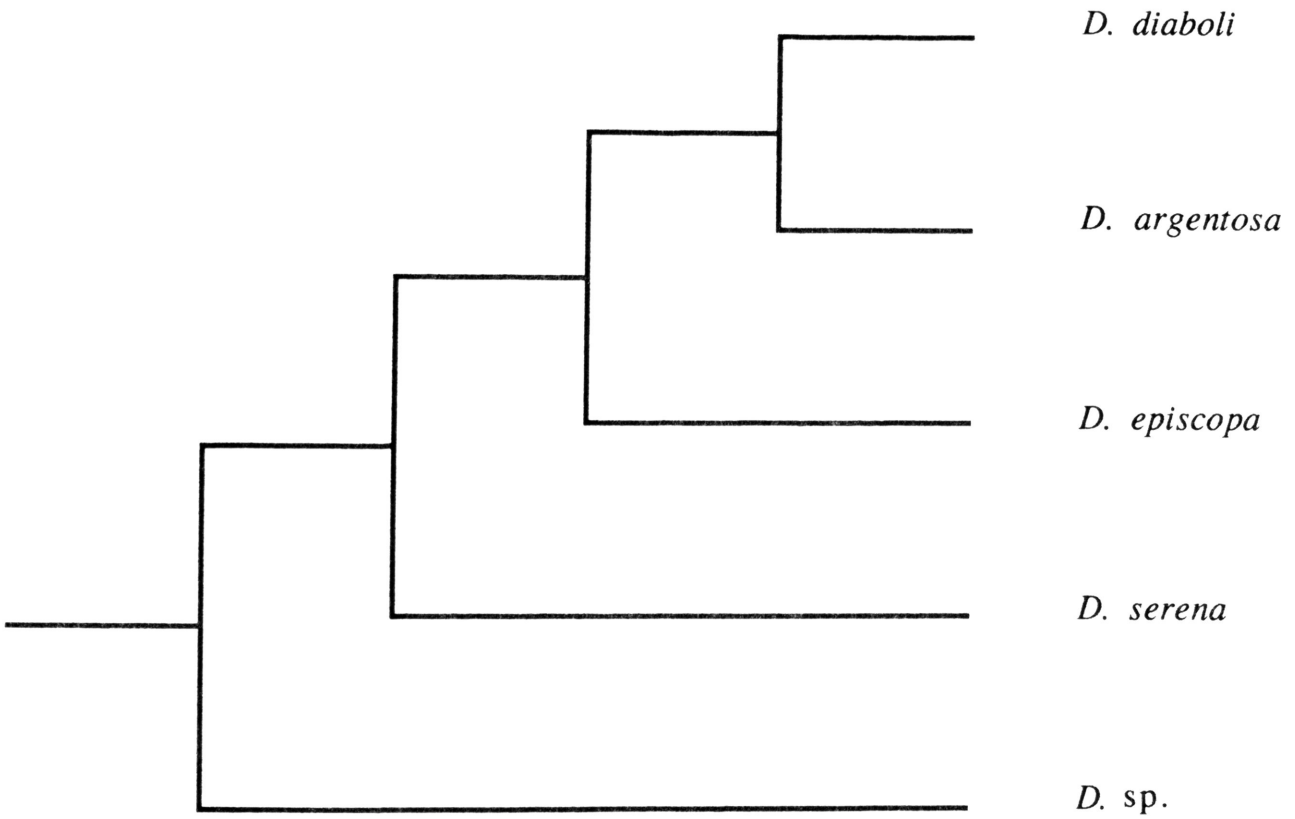

Figure 1: Phylogenetic hypothesis for the *Dionda episcopa* complex as determined by allozymes (Mayden et al., 1991).

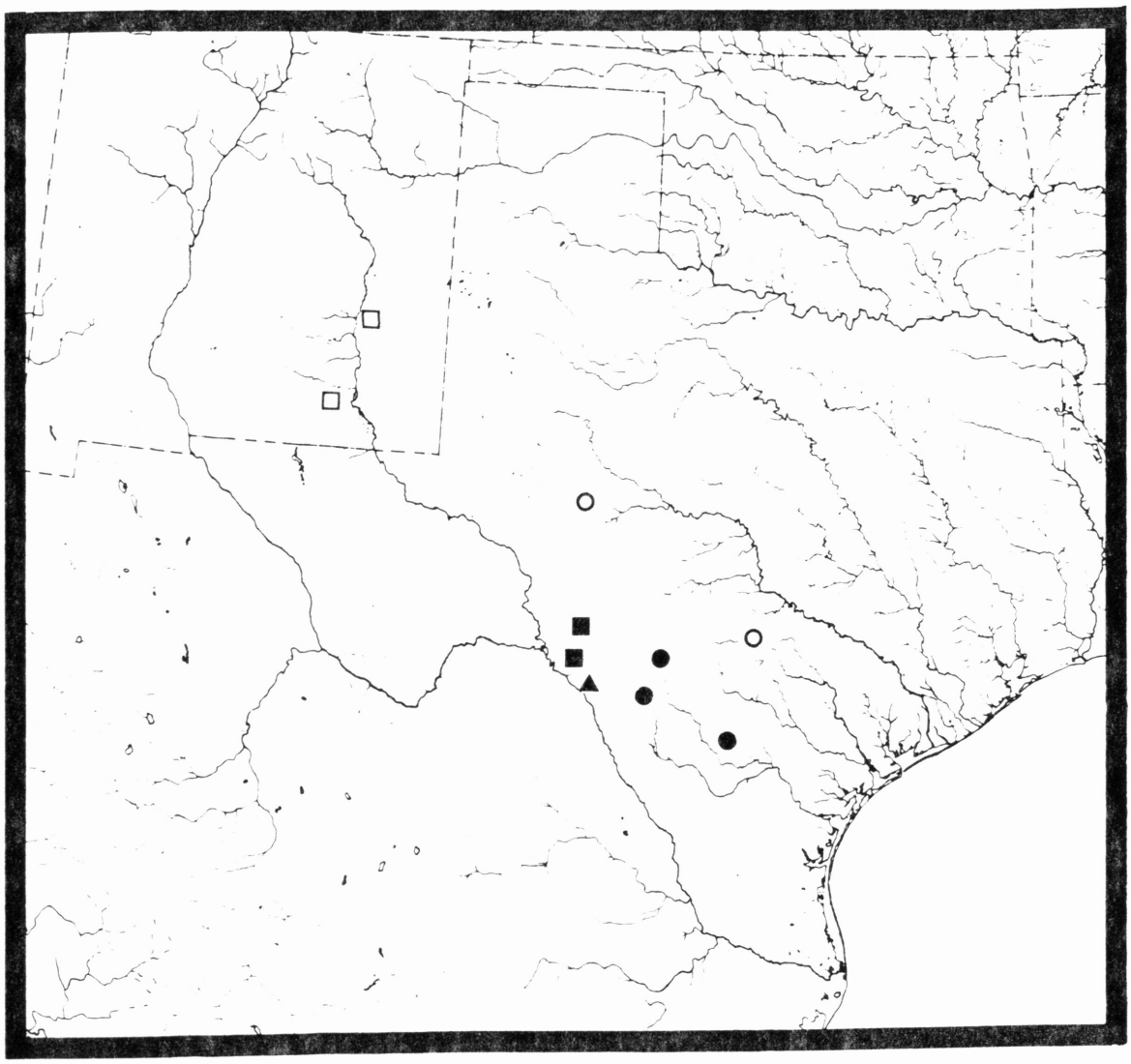

Figure 2: Sampling localities for *Dionda* material:
■ = *D. argentosa*, ▲ = *D. diaboli*, □ = *D. episcopa*,
● = *D. serena*, ○ = *D.* sp. (Guad.-Col. R.).

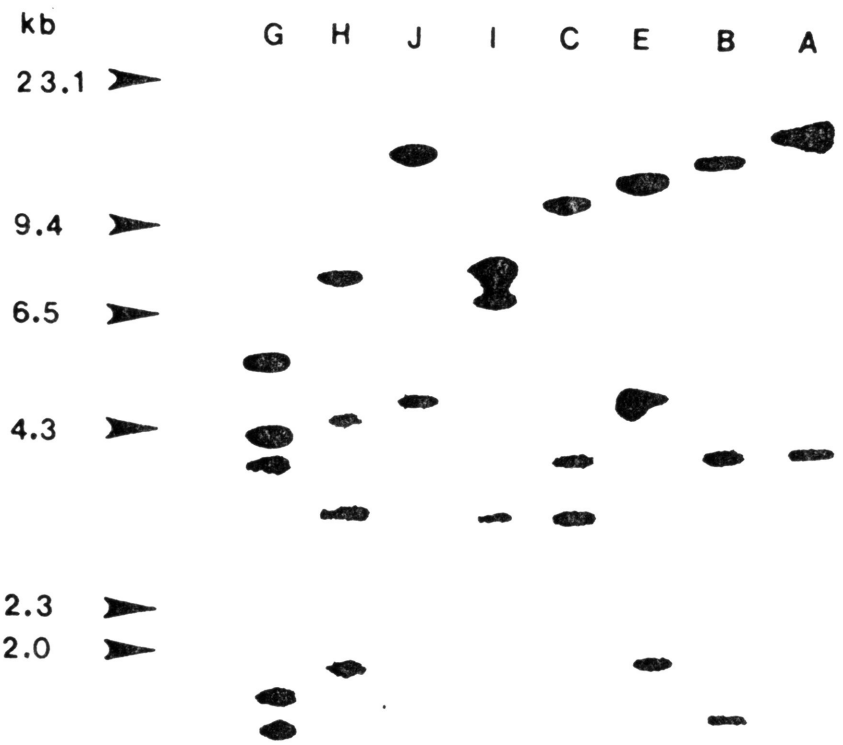

Figure 3: Side-by-side comparisons of *Bgl* II single digestions of *Dionda* mtDNA.

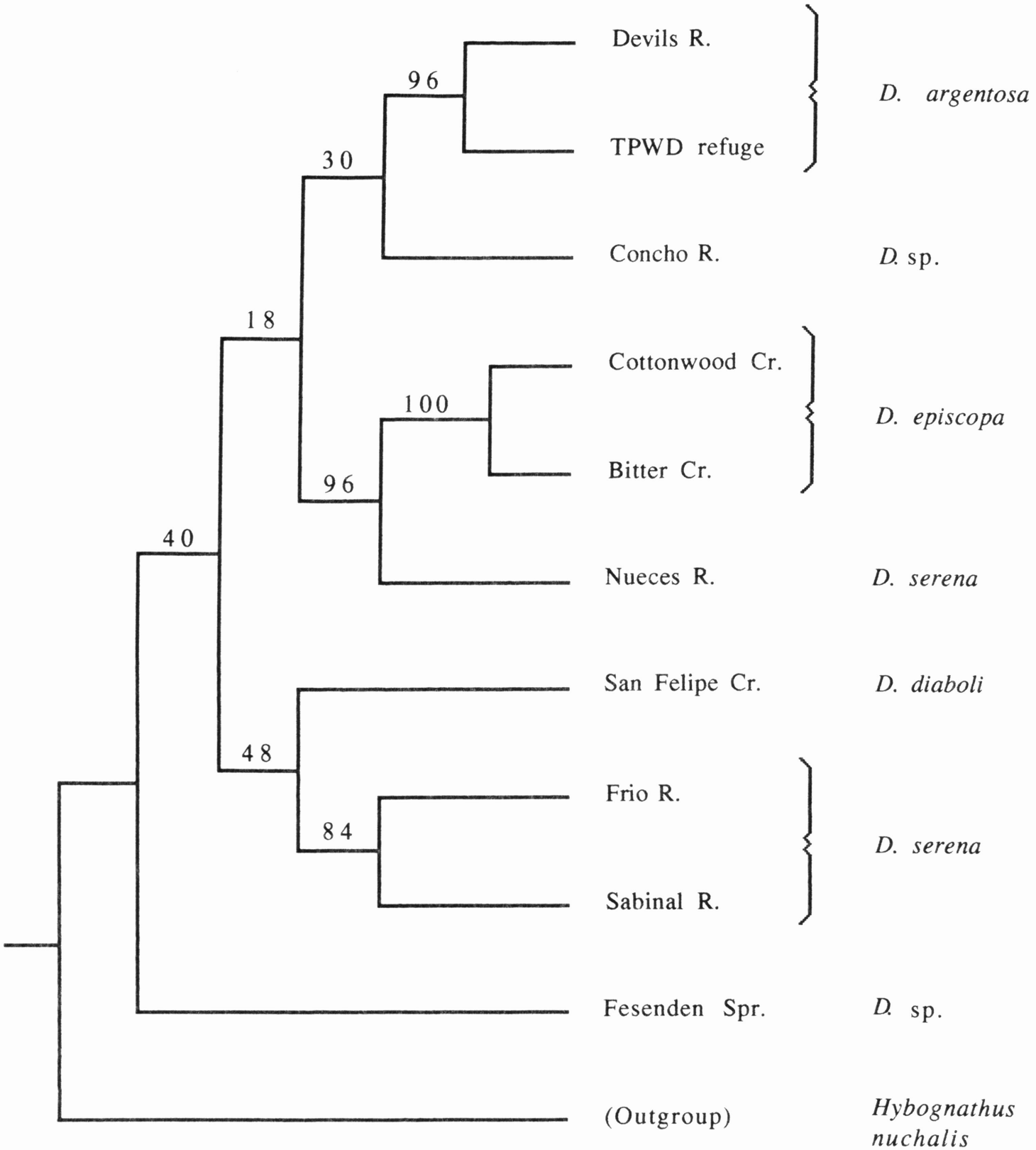

Figure 4: Phylogenetic tree for the *Dionda episcopa* complex created using PAUP. Numbers on the nodes represent bootstrap values.

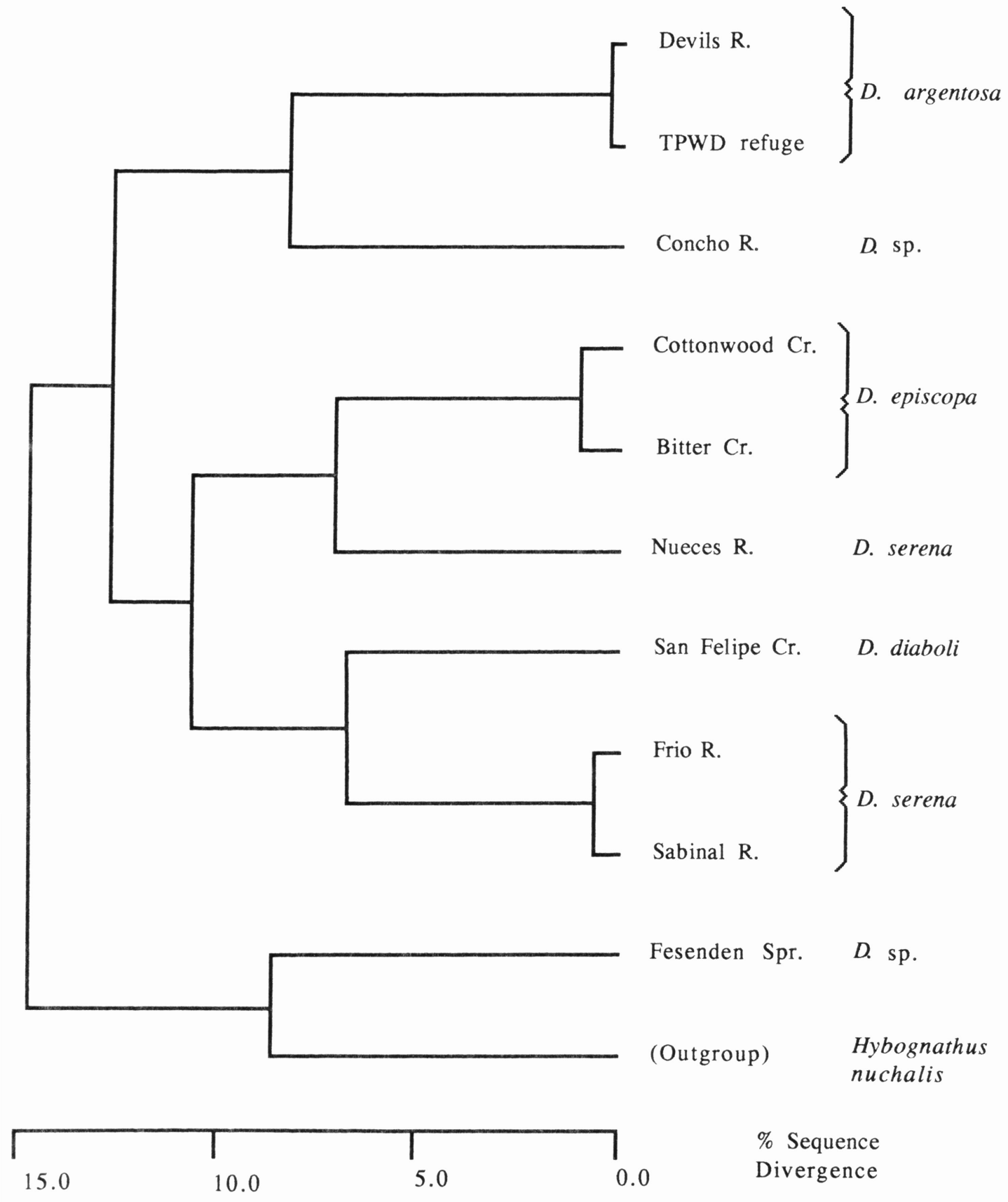

Figure 5: Phenogram for the *Dionda episcopa* complex created using UPGMA.

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