

**RESULTS OF PRIOR FUNDING: Karen Renzaglia. NSF Award DEB-9527735, "Spermatogenesis in pteridophytes": ultrastructure, differentiation and phylogeny," 2/96 to 2/01, \$140,000.** Our major research findings fall into two categories: (1) comparative information on cellular development and structure in land plants and (2) contributions to clarifying evolutionary trends and resolving phylogenetic relationships among basal embryophytes. We have provided detailed descriptions of sperm cell architecture and cellular development in pteridophytes, bryophytes, green algae and seed plants. Our work reveals that structural and developmental complexity in plant sperm cells is unsurpassed in any other group of organisms. The role of cytoskeletal elements is complicated, and our studies have systematically identified the involvement of these proteins during spermatogenesis in representatives of the major plant groups. We have generated new data, assembled published data and analyzed one of the most comprehensive data bases of both morphological and molecular data associated with the phylogeny of land plants. Since 1995, 27 published manuscripts were generated, with six undergraduate co-authorships (1-27, undergraduates in bold). The nine REU-supported undergraduates at SIUC who participated in this research received numerous small grants and awards for research and travel. One doctoral student, and three master's students worked on plant spermatogenesis since 1995.

## PROJECT DESCRIPTION

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### I. What is new about this proposal?

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This proposal represents a revision of one submitted to the panel in January 2002 (DEB-0212649). The reviewers of the prior proposal strongly support the objectives of the research and agree that the general approach to achieving the objectives is sound. Nevertheless there are a number of criticisms that collectively suggest that although the project is sound in concept, it may be too broad in focus and that lack of detail in application of techniques may hinder execution of the project as described. Thus, the current proposal retains the central objectives but differs from the previous proposal in the incorporation of new molecular and morphological data that allow us to fine tune/focus our goals and better demonstrate the utility of our approaches to molecular and morphological data collection and analysis. We have addressed all of the specific criticisms of reviewers and we have demonstrated stronger ties with other research programs in our area of interest, thereby increasing the impact of our work.

Specific criticisms of the prior submission can be grouped into three broad categories: 1) scope of research, 2) preliminary data, and 3) methods of analysis. Below we lay out the major changes in each of these areas:

**1. Scope of research:** We agree with the reviewers who expressed concerns about the ambitious nature of the project. Specifically, they questioned the proposed intensive evaluation of infra-specific variability in the cosmopolitan *Phaeoceros laevis* complex. These reviewers noted that too many questions at too many levels were being addressed, thus jeopardizing the successful completion of the project. We have decided that given the current state of taxonomic confusion in the hornworts, the need to address basic taxonomic issues and develop a well-resolved phylogeny of the group is of paramount importance. A program that focuses on these fundamental concerns/deficiencies in systematic biology is sufficiently ambitious without addressing questions of cryptic speciation and population scale genetic diversity. Hence we have removed that component from the current proposal with the intention of pursuing these questions in future studies; results from our proposed study will form the basis for such continued work.

**2. Preliminary data:** Continued collection of molecular and morphological data since the prior submission has yielded a wealth of new data that in turn have provided new insights into investigating hornwort systematics. **Molecular sequence data:** Several reviewers were skeptical that partial *nad2* (mtDNA) and ITS (nDNA) sequences would be informative in addressing taxonomic questions within hornworts. *nad2* is readily amplified from hornwort samples enabling us to expand sampling and to demonstrate unequivocally the usefulness of this gene in elucidating hornwort relationships at different hierarchical levels. Because we are no longer focusing on infra-specific questions, we propose to omit ITS sequences and replace them with sequences from the 3' end of the *nad2* gene, encompassing an additional 1.6 kb

portion of an intron and 500 bp of protein coding sequence. The combination of 18S (nDNA), *rbcL* (cpDNA) and the *nad2* gene (mtDNA), including 3.0 kb of intron sequences, will provide resolution of familial, generic and specific relationships among the hornworts. **Morphological data:** A recent collaborative research visit by J. Duff and C. Cargill to K. Renzaglia's lab resulted in substantial collection and analysis of taxonomic, morphological and ultrastructural data. Our observations reinforced the contention that the taxonomy of this group is in a state of confusion and that modern morphological data are required to clarify taxonomic affinities and to identify diagnostic features of species and genera. Examination of poorly-known taxa from New Zealand, Australia and Panama, many of which were contributed and processed by J. Duckett, R. Ligrone and N. Salazar, resulted in 1) revised concepts of criteria for taxonomic delineation and 2) exciting new hypotheses of character evolution within the group. We have focused our morphological studies to ensure that examination of the taxonomic characteristics and assessment of ultrastructural features are accomplished for each specimen and that all members of the team are able to collect and/or analyze these data. With an integrated collaborative approach such as this we are able to effectively and efficiently address taxonomic questions and test evolutionary hypotheses.

**3. Methods of analysis:** **a) Phylogenetic Analyses:** Although still debated, it is generally accepted that the total evidence approach is best suited for addressing deep phylogenetic questions such as we raise within the hornworts (28-31). Hence, we will seek to combine all data sets including morphological and molecular. Because we are interested in relationships at multiple hierarchical levels, we will still employ, as previously proposed, the conditional-combination approach as it affords greater flexibility at multiple scales. **b) Outgroup selection:** We have identified three charophycean algae, two liverworts, two mosses, one lycophyte and one fern from which primary data will be collected. The selection of putatively basal taxa will ensure adequate sampling of these key lineages. **c) Sequence alignment:** Only the *nad2* intron sequences present alignment challenges. We will do analyses of sequences for which only unambiguous alignment can be achieved but we argue that positions requiring alignment gaps should be included and either treated as missing data or coded as a fifth character state. **d) Biogeography:** Few biogeographic evaluations of bryophytes have been made regardless of methodology (32). Hence, we propose to apply two common methods of biogeographic analysis to our data with the goal of producing a general view of global geographic patterns while assessing specific questions related to southern hemisphere biogeography. We will use standard methods that have been tested on other groups in an effort to provide fundamental data on bryophyte biogeography to a community typically focused on patterns of angiosperm diversity.

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## II. INTRODUCTION AND OBJECTIVES

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As the oldest living lineages of land-dwelling organisms, the bryophytes (mosses, liverworts and hornworts) represent pivotal groups for evaluating early patterns of terrestrial biodiversification. While there has been a recent resurgence of interest in moss and liverwort biodiversity and taxonomy (32,33, and 34 and refs. cited therein), one group of bryophytes, the **hornworts**, has remained relatively overlooked. Hypothesized to be the basal most land plants (7,12,13,35-42), the hornworts (anthocerotes) are a small group (5-12 genera with 100-150 species worldwide) of morphologically distinct, seemingly simple land plants. Many structural features of these plants represent adaptations to life on land that are the products of deep divergences and thus are unparalleled in other groups of plants (6). Even with unique innovations, the hornworts possess characters that are algal-like, liverwort-like, moss-like, and yet others that are similar to vascular plants (e.g., continued sporophyte growth, embedded archegonia). Some of these features, especially chloroplast ultrastructure and sperm cell architecture, are viewed as plesiomorphic (35) while others are considered advanced characters among bryophytes (e.g., complicated sporophyte, presence of stomata) (42). It is because of the unique structural innovations and the curious shared characteristics with other plant and algal lineages that this ancient group of land plants is particularly intriguing from an evolutionary perspective.

While hornworts clearly represent a monophyletic group, taxonomic boundaries and interrelationships among species and genera remain obscured (6). Existing classification schemes are incongruent and conflicting generic concepts recognize from five to nine, totaling 12, genera (43-46). Although diagnostic morphological features exist in some cases, other critical characters for delineation of

genera and species show considerable variation. The inconsistent use of characters to delineate taxa and the paucity of fundamental developmental, ultrastructural and biochemical data further confuse hornwort taxonomy. Confounding this is the fact that the anthocerotales are the **only** major group of land plants for which a molecular study has not yet been published at **any** taxonomic level. Due to the absence of molecular phylogenetic studies and the lack of informative morphological data, it is virtually impossible to evaluate hypothesis of adaptive evolution within the hornworts and to place these innovations into the broader context of land plant evolution. Clearly, new data and new insight into analyzing existing data are required to clarify the systematics and evolution of this important, ancient plant lineage.

We propose a **global examination of hornworts** that will develop a portrait of the biodiversity, phylogeny and biogeography of this plant assemblage. Our collaborative research will provide the necessary data on genetic/morphological diversity and geographic distribution to achieve the following:

**1) Establish a robust estimate of hornwort relationships.** To achieve this goal we will conduct analyses of multiple molecular data sets and comprehensive data sets that include morphological, ultrastructural, functional and developmental characters. Gene sequences will be generated from all three genomes. Morphological data will encompass features that are emphasized in taxonomic treatments and new ultrastructural data that enable within hornwort comparisons as well as more global comparisons with algal and other land plant groups. This goal is essential to assess morphological character evolution and to accomplish the remaining goals.

**2) Produce a revised classification of the hornworts.** Rather than simply providing phylogenetic relationships to the systematics community, we feel strongly that a modern reclassification of the hornworts should result from our efforts. We will approach the classification from a cladistic perspective applying clades derived from phylogenetic analyses to the organisms included in our study. This approach will clarify the presently poorly defined familial and generic boundaries.

**3) Identify patterns of hornwort diversification.** To gain a greater appreciation for the spatial and temporal patterns of hornwort diversification, we propose to examine worldwide biogeographic patterns. The antiquity of hornworts presents unique opportunities to test biogeographic hypotheses of vicariance versus recent colonization, typically generated from studies of vascular plants and animals. We propose to **(a)** identify general global biogeographic patterns, and **(b)** examine specific biogeographic questions involving the Australasian landmasses and relate them to the large body of literature for the region.

To address the above goals, we request three years of support to conduct two highly integrated and collaborative projects. The first (J. Duff) employs molecular phylogenetic methodologies whereas the second (K. Renzaglia) involves comparative morphogenetic and ultrastructural studies. Integral to the project are further collaborations with Chris Cargill from the Australian National Herbarium at the Center for Plant Biodiversity Research (CANB) and Jeff Duckett from the University of London (see attached letters). As experts on hornwort taxonomy and morphology, C. Cargill and J. Duckett have and will collect intensively in the Australasian landmasses, Southeast Asia and Africa and will play integral roles in morphological data collection and analyses. Additionally, we have called on collaborators from around the world, including Jiro Hasegawa (Japan), Noris Salazar (Panama), Yelitza León (Venezuela), Benito Tan (Singapore), Roberto Ligrone (Italy), A. K. Asthana (India), S. C. Srivastava (India), Kevin Vaughn (Mississippi), Wilson Taylor (Wisconsin), David Hanson (New Mexico) and Paul Davison (southeastern USA), to assist in specimen collection and to collaborate on our studies (see attached letters).

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### III. BACKGROUND

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**A. Unique Hornwort Characters:** The peculiar structure and development of the sporophyte separates hornworts from all other land plants. This generation consists of a **long cylindrical sporangium** that **perpetually elongates** from a basal meristem and produces spores in a **non-synchronized** fashion. The sporophyte consists of an epidermis with or without **stomata**, an assimilative tissue, sporogenous tissue and a **central columella**. Although similar in appearance to those of mosses and tracheophytes,

hornwort stomata apparently **do not close**, i.e., once open, they remain that way. The sexual phase or gametophyte generation, is a simple flattened thallus or ribbon with internal chambers and cavities that house sex organs and **colonies of *Nostoc***, a cyanobacterial symbiont. Peculiar to this group is the abundant production of **mucilage** by virtually any cell. Indeed, reproductive organs and vulnerable tissues are enclosed in **internal, mucilage-filled chambers** that provide protection against desiccation, ionizing radiation and mechanical damage (7). At the cellular level, hornworts are unique among land plants in the possession of **solitary chloroplasts with pyrenoids**, features that are shared with green algae (47). **Morphological variability among genera** is primarily seen in reproductive features such as the architecture of the sporophyte, spores, pseudoelaters, antheridia and antheridial chambers, but generic differences are also seen in the thallus structure, and number and microanatomy of chloroplasts.

**B. Questionable Hornwort Affinities:** Hornworts are in the center of a heated debate over land plant evolution (7,36,40,48-50). Recent molecular and morphological evidence suggests the hornworts are the oldest living lineage of land plants (7,12,13,35-42). An equally viable hypothesis identifies liverworts as the basal most lineage of land plant, with either hornworts or mosses sister to the vascular plants (48-54). Although resolution of this fundamental question in evolutionary biology is not our focus, further characterization of hornwort phylogeny and character evolution is essential in providing a complete picture for interpretation of the evolutionary events that accompanied land colonization. For example, if the directionality of evolution in structures such as stomata and chloroplasts is clarified within hornworts, then the homology with similar structures in other plant and algal groups can be ascertained.

**C. Problematic Hornwort Taxonomy and Classification:** Cladistic analyses involving morphological features of a limited number of samples have been conducted on hornworts (44,51), and some of the resultant phylogenetic hypotheses have been used to develop classifications schemes (43-46). Of the twelve proposed genera of hornworts, only five have gained wide recognition, namely *Anthoceros*, *Phaeoceros*, *Notothylas*, *Megaceros* and *Dendroceros*. As evidenced by the lack of agreement on classification schemes, the hierarchical organization of hornwort taxa remains equivocal and taxonomic boundaries are poorly resolved. Mishler and Churchill (51) noted the problematic relationships within and among hornwort genera and stated that almost all of the apparent homoplasy they detected in their morphological analysis of bryophytes involved the hornworts!

An evaluation of the divergent interpretations of the evolutionary origins of *Notothylas* and the conflicting views on the placement of this genus among hornworts highlights the critical need for a modern taxonomic revision of the hornworts. With a relatively simple, unelaborated sporophyte, *Notothylas* may represent 1) an apomorphic form that has undergone extensive evolutionary reduction or 2) a plesiomorphic, unspecialized condition. Schuster (42) stated that, "The consensus (among bryologists) has been that a reduced genus, derived from *Anthoceros* s. str. is at hand." He further noted that the generic characters are spurious or minor and the circumscription of the genus problematic. Proskauer (55) corroborated this view and speculated that *Notothylas* may be a possible polyphyletic derivative from *Anthoceros*-like forms. In contrast, a basal position for *Notothylas* was proposed by Mishler and Churchill (51) and is supported by characters interpreted as retained plesiomorphies, e.g., indehiscent sporophyte, determinate sporophyte growth, and rudimentary or absent columella (45,46,56).

There appears to be some agreement on evolutionary affinities between *Anthoceros* (= Schuster's *Aspiromitus*) and *Phaeoceros* (= Schuster's *Anthoceros*), and between *Dendroceros* and *Megaceros*, albeit all four of these taxa have been segregated into multiple genera and subgenera (42-46). Challenging the putative close relationship between *Phaeoceros* and *Anthoceros* and the hypothesized basal position of *Notothylas*, our preliminary molecular analyses of both *rbcL* and *nad2* sequences provide strong support for a monophyletic *Anthoceros*, a clade that is basal to all other hornworts (Figure 1 and discussion below). Sister relationships between *Dendroceros* and *Megaceros* and between *Phaeoceros* and *Notothylas* are supported, while the monophyly of *Phaeoceros* is called into question. When morphological data are evaluated in light of this hypothesis, a new, surprising interpretation of character evolution emerges (see discussion in section V.B.). Clearly, a comprehensive re-evaluation of hornwort systematics and evolution is warranted.

#### **D. Why have hornwort biodiversity, phylogeny and biogeography not been reexamined?:**

Evolutionary biologists and bryologists face a number of difficulties in studying hornworts. First, the acquisition of specimens and identification of taxa are major obstacles. To overcome these problems, we have networked with many of the world experts on hornworts and they are eager to participate in this study (see attached letters). For the first time, morphological features of multiple specimens will be examined in detail and molecular analyses will be performed. This kind of comprehensive approach will finally enable clarification of hornwort taxonomy, which in turn is necessary for examination of biogeography. Secondly, the preservation of hornwort tissue for electron microscopy and the application of molecular methodologies have proven problematic in the past. Through problem solving, we have systematically resolved the difficulties with protocols in our laboratories, and we are now in an ideal position to rapidly accumulate and analyze data.

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#### **IV. PRELIMINARY RESULTS**

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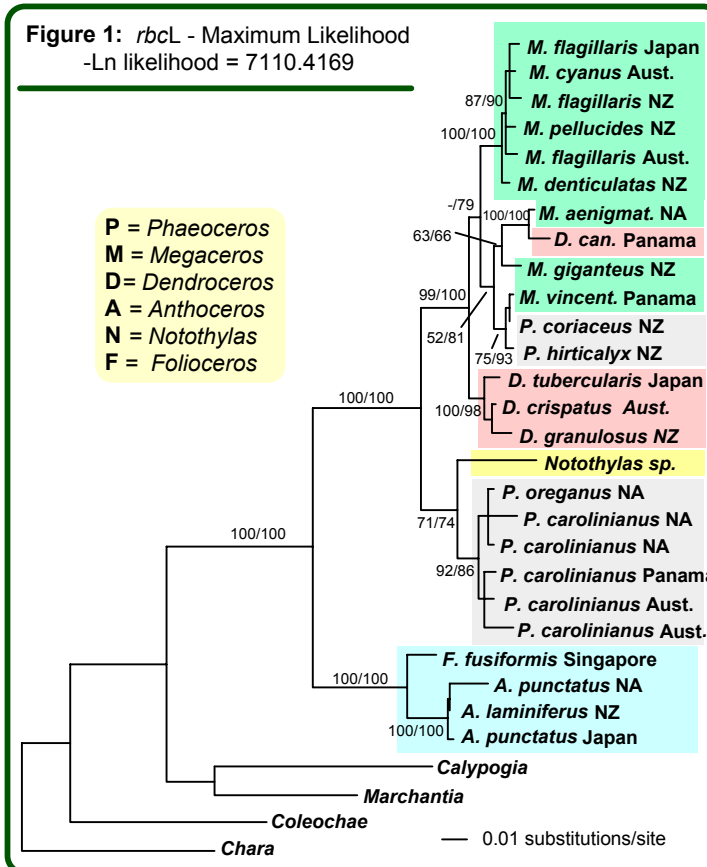
To develop the most effective approach to a comprehensive re-evaluation of hornwort systematics and biogeography, we acquired collections of 52 samples representing 31 putative species of hornworts from Central America, North America, Southeastern Asia, New Zealand and Australia. Preliminary studies included screening of candidate genes for phylogenetic reconstruction, phylogenetic analysis of molecular sequence data, and SEM, TEM and light microscopic work on a large number of the specimens. Below we present an overview of our preliminary molecular and morphological studies as a way of introducing the data that shaped our research plan.

**Molecules:** Table 1 summarizes the results of parsimony analysis of 26 *rbcL* sequences, 12 of the 5' end of *nad2* (ca. 1900 bp) and 11 18S sequences, all of which demonstrate utility in resolving phylogenetic relationships among hornworts. Further analyses of the *rbcL* and *nad2* sequences included maximum-likelihood analysis (see Figures 1 and 2 for details of analyses).

The following hypotheses and observations may be made from the analyses in Figure 2 that are relevant to the goals of the proposed research: **1)** Genetic divergence among putative genera is substantial, validating the suggestion that there are deep divergence points among extant taxa; **2)** The taxa fall into well-supported clades that show reasonable agreement with current concepts of generic limits, but the hypothesized interrelationship of these taxa is novel; **3)** *Anthoceros* is basal to the remaining hornworts; **4)** The position of *Phaeoceros hirticalyx* and *P. coriaceus* in the *Megaceros* and/or *Dendroceros* clades calls into question the current concepts of generic limits; **5)** The placement of *Anthoceros lamuniferus* within *A. punctatus* and *Phaeoceros oregonos* (*rbcL*) within *P. carolinianus* challenges concepts of species limits; **6)** Affinities of *Nothoceros* (*N. giganteus*) and *Folioceros* (*F. fusimormis*, *nad2*) with *Megaceros* and *Anthoceros*, respectively, suggest elevation of these segregates to generic status is unwarranted. Clearly, many more taxa are necessary to define familial, generic, and subgeneric limits.

**Morphology:** Our preliminary investigations highlight the importance of collecting fundamental developmental and ultrastructural information for all specimens. Phylogenetic analyses based on these data will provide a more thorough understanding of biocomplexity within the hornworts and will enable elucidation of historical modifications at the cell, tissue and organ levels. To illustrate how **taxonomic problems may be solved with microscopic examination combined with molecular analyses**, we present the results of a recent study of *Dendroceros caniculatas* from Panama. This species superficially resembles other species of *Dendroceros* in habit, but upon close examination exhibits critical taxonomic characteristics shared with *Megaceros*, the most notable of which are the possession of green single-celled spores (not multicellular spores typical of *Dendroceros*) and pyrenoid-lacking chloroplasts. Not surprisingly, analysis of *rbcL* sequences reveals that *D. caniculatas* nests within the *Megaceros* clade (Figure 1). Congruence of morphological and molecular data results in resolution of the placement of this taxon among hornworts and provides clarification of diagnostic morphological features for the *Megaceros* and *Dendroceros* clades.

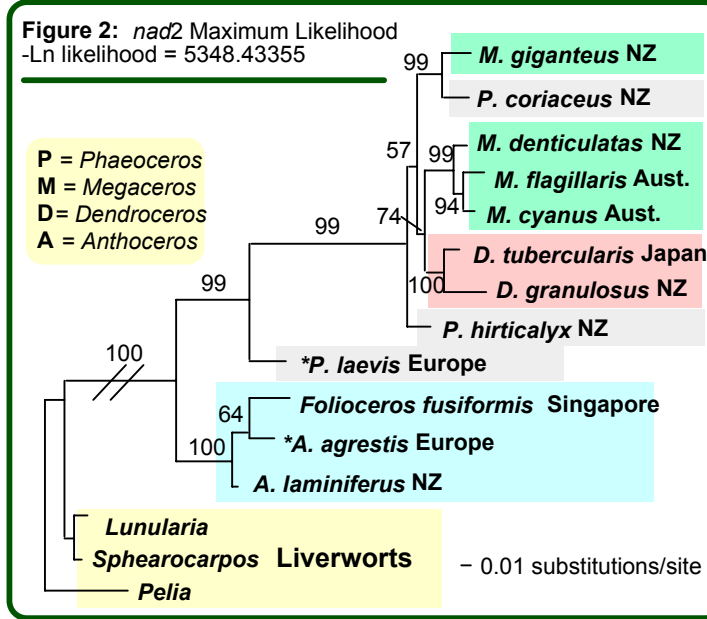
Preliminary morphological observations **when viewed in conjunction with results of molecular phylogenetic analyses** also point to two **new interpretations** of relative timing and directionality of **structural evolution** within hornworts (**Figures 1 and 2**). As our working hypothesis, the preliminary molecular phylogenies present novel relationships that are highly intuitive when morphological characters are reevaluated, thus providing interesting new insights into character evolution. **1) Stomatal evolution:**



**Table 1.** Comparison of maximum parsimony analysis for the 18S, *rbcL*, and *nad2* genes. For the *nad2* the protein coding and intron sequence are grouped and only completely unambiguous sequence were compared (see above)

	18S	<i>rbcL</i>	<i>nad2</i>
# of samples	14	30	15
Trees	9	144	1
Length	285	453	481
Characters	1805	1351	1604
Uninformative	188	109	145
Informative	59	210	240
Clexcluding uninformative	0.784	0.712	0.897

**Figure 1.** Results of ML analysis of 1351 bp of *rbcL* sequence using the HKY model and a ti/tv ratio of 2. Numbers at nodes represent 100 ML and 1000 Maximum parsimony bootstrap replicates. Nodes with no number had values less than 75 for both. Additional analyses included additional liverworts and mosses but had no effect on the hornwort topology.

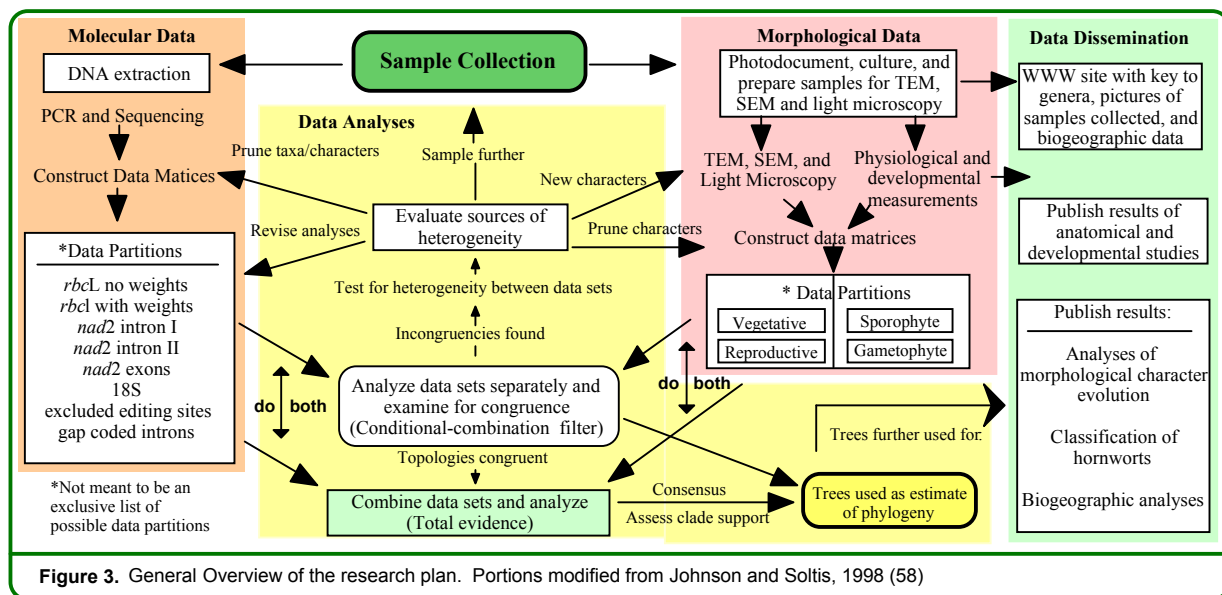


**Figure 2.** Results of ML analysis of the *nad2* gene including 510 bp of the protein coding sequence and 1094 bp of aligned intron sequence that were unambiguously aligned. ML was done under the same conditions as above which were also used with *nad2* by Beckert et al. (57). Ca. 520 bp were eliminated from the analysis. Only the protein coding (510 bp) and 350 bp of the intron were aligned with the outgroup liverworts. Numbers at nodes represent 100 bootstrap replicates. \* denote Genbank sequences. *M. giganteus* = *Nothoceros giganteus*. Of the 520 eliminated bases most could be readily aligned with one or more samples exhibiting indels. If indels were scored as a fifth character there would be 57 potential sites after excluding all obvious simple sequence repeats which are to be avoided.

Analysis of *rbcl* and *nad2* sequences (Figures 1 and 2) suggests that stomata are plesiomorphic (present in *Anthoceros* and *Phaeoceros*) and that they have been lost independently at least three times during hornwort evolution (*Notothylas*, *Megaceros* and *Dendroceros*). The presence of stomata in *Phaeoceros coriaceus* and *P. hirticalyx* (unpublished data), a potentially basal taxon in the *Megaceros* clade raises the possibility that ultrastructural examination of these stomata will elucidate structural changes that have accompanied stomatal loss in the hornwort lineage. **2) Chloroplast evolution:** Although the sporophytes of *P. hirticalyx* and *P. coriaceus* show key features in common with *Phaeoceros* (non-spiraled pseudelaters and stomata), the chloroplast are clearly diagnostic of *Megaceros*, i.e., they lack a pyrenoid and may number more than one per cell. This supports the interpretation that pyrenoid loss and increase in chloroplast number occurred prior to evolutionary changes in the sporophyte, and that, contrary to current opinion, features of the chloroplasts may be more informative in defining genera than sporophytic characteristics.

## V. RESEARCH PLAN

Figure 3 and the descriptions below provide an overview of our proposed research program that involves the integration of multiple research approaches, techniques and methods of analyses.



**A. Sample acquisition, proposed collection and preparation:** We will obtain samples (see attached letters) from around the world that represent the entire spectrum of hornwort diversity. These will include species of all the named generic segregates and subgenera (Figure 4). In order to obtain the complete suite of morphological data, including ultrastructural information, living specimens are vital. Consistency in the collection of morphological and molecular data is crucial. We will strive to obtain all data from a single source, investigating the morphological and ultrastructural features of all the specimens used for molecular analyses. To insure that we have sampled as much of the genetic diversity as possible, we will seek to obtain DNA from herbarium specimens of crucial taxa only if living specimens are impossible to obtain. Of the approximately 120 total species we will gather at least 70 species, representing those with expansive and restrictive ranges. Especially intensive sampling will be conducted from the Australasian regions and India where endemism and species diversity are particularly great. Specimens will be cultured at Southern Illinois University, the University of Akron and the Australian National Herbarium, and voucher specimens will be deposited at CANB, with duplicates donated to the herbarium at the Missouri Botanical Garden (MBOT). Collection data, including sample photos will be documented on our hornwort web site (<http://www.uakron.edu/biology/hornworts/hornworts.html>).

**B. Morphological/ ultrastructural studies:** A major component of this project involves the accumulation and interpretation of morphological data. Our studies are designed to provide reliable contemporary morphological data that will correct errors, clarify ambiguity and augment information available in the literature, hence original data will be collected for all taxa investigated. These data will consist of 1) gross anatomical and morphological features that can be derived from light microscope observation of living and dried material and 2) ultrastructural, developmental and physiological data that require tissue preparation and observation in the TEM, SEM, fluorescence or light microscope.

**Figure 4.** Hornwort species targeted for this study. The following subgenera (a-e) were recognized as genera by: (a) Hassel de Menendez, 1988 (44), (b) Bharadwaj, 1971 (59), (c) Piipo, 1993 (60), (d) Hassel de Menendez 1986 (61), and (e,f) Haegawa (1994 (46). Locations: W=Worldwide E=Europe A=Africa SEA=Southeast Asia CA=Central America SA=South America P=Paletropica NZ=New Zealand I=India PB=Panboreal Aust=Australia NA=North America J=Japan WI=West Indies. Bold are species we have samples prepared for morphological and molecular analyses. Asterisks denote species for which we have more than one sample.

<b>Anthoceros L.</b>	<b>Location</b>	<b>Notothylias Sull.</b>	<b>Location</b>
<i>Anthoceros</i>		<i>Notothylias</i>	
<b>A. punctatus L.*</b>	W	<b>N. orbicularis (Schwein.) Sull*</b>	PB
<b>A. agrestis Paton*</b>	E	<i>N. breutellii</i> (Gott.) Gott.	WI,NA
<i>A. expansus</i> Steph.	A	<i>N. javanicus</i> Sande Lac.) Gott.	SEA
<i>A. fusiformis</i> Aust.	NA,J	<i>N. temperata</i> Haseg.	EA
<i>A. pinnatus</i> Steph.	A	<i>N. indica</i> Kash.	I
<i>A. cavernosus</i> Steph.	WI	<i>N. galapagensis</i> Howe	CA
<i>A. erectus</i> Kash.	I	<i>Depressisporae</i>	
<i>A. glandulosus</i> L. & L.	I	<i>N. depressisporae</i> Haseg.	J
<i>A. angustus</i> Steph.	SEA	<i>N. dissecta</i> Steph.	I,CA
<b>A. laminiferus Steph.</b>	NZ		
<i>A. myriandroecium</i> Steph.	A		
<i>A. tuberculatas</i> Lehm.	CA		
<i>A. hispidus</i> Steph.	SA		
<i>A. simulans</i> Howe	CA		
<i>Sphaerosporoceros</i> (a)		<b>Phaeoceros Prosk.</b>	
<i>A. adscendens</i> L. & L.	NA	<i>Phaeoceros</i>	
<i>A. macounii</i> Howe	NA	<b>P. laevis (L.) Prosk*</b>	W
<i>A. capricornii</i> Cargill & Scott	Aust	<b>P. carolinianus (Michaux) Prosk.*</b>	W
<i>Folioceros</i> (b)		<b>P. oreganus (Aust.) Hassel</b>	W
<b>F. fuciformis (Mont.) Bharadw.</b>	Aust,SEA	<b>P. morhii (Aust.) Hassel</b>	NA
<i>F. appendiculatas</i> (Steph.) Haseg.	SEA	<i>P. pearsonii</i> (Howe) Prosk.	NA
<i>F. amboinensis</i> (Schiffn.) Piippo	SEA	<i>P. parvulus</i> (Schiffn.) Haseg.	SEA
<i>F. dilatatus</i> (Steph.) comb. nov.	A	<i>P. gemmifer</i> (Horik.) Haseg.	J
<i>F. apiathynus</i> (Steph.) Hassel	SA	<i>P. hallii</i> (Aust.) Prosk.	NA
<i>F. indicus</i> Bharad.	I	<b>P. coriaceus (Steph.) comb. nov.</b>	NZ
		<b>P. hirticalyx (Steph.) Haseg.</b>	NZ
		<i>P. fimbriatus</i> (Gott.) Gradst.	SA
		<i>P. foveatus</i> Hase.	SEA
		<i>P. bulbiculosus</i> (Brotero) Prosk.	E,A
		<i>Mesoceros</i> (c)	
		<i>M. mesophoras</i> Piippo	SEA
		<i>M. porcatus</i> Piippo	SEA
		<i>Leiosporoceros</i> (d)	
		<i>P. dussii</i> (Steph.) Hassel	WI
		<i>Hattorioceros</i> (e)	
		<b>P. striatisporus (Haseg.) Haseg.</b>	I
		<b>Megaceros Camp.</b>	
		<i>Megaceros</i>	
		<b>M. denticulatas (Lehm.) Steph.*</b>	NZ
		<i>M. leptohymeius</i>	
		(Hook. f. & Taylor.) Steph	NZ
		<i>M. arachnoideus</i> (Steph.) Steph.	NZ
		<b>M. pellucidus</b>	
		(Colenso) E.A.Hodgs.	NZ
		<b>M. cyanus Nom. Herb.*</b>	Aust
		<b>M. vincentianus (L.&amp;L.) Steph.*</b>	CA
		<b>M. aenigmaticus Schust.*</b>	NA
		<b>M. flagellaris (Mitt.) Steph.*</b>	P
		<i>M. fusiformis</i>	J
		<i>Nothoceros</i> (f)	
		<b>M. endiviaefolius Mont.</b>	SA
		<b>M. giganteus (L.&amp;L.) Haseg.</b>	NZ
<b>Dendroceros Nees</b>			
<i>Dendroceros</i>			
<b>D. crispus (Sw.) Nees*</b>	Aust		
<b>D. japonicus Steph.</b>	J		
<i>D. acutilobus</i> Steph	SEA		
<b>D. javanicus (Nees) Nees</b>	SEA		
<b>D. tubercularis Hatt.</b>	J		
<i>D. borbonicus</i> Steph.	SEA		
<b>D. ganulatus Mitt.</b>	SEA		
<b>D. canaliculatus Pagan*</b>	CA		
<i>D. validus</i> Steph.	NZ		
<i>Apoceros</i>			
<i>D. difficilis</i> Steph.	SEA		
<i>D. pedunculatus</i> Steph.	SEA		
<b>D. cavernosus Haseg.</b>	SEA		

In order to focus our morphological studies, we have identified characters that are specific to hornworts and that are designed to approach questions relating to generic boundaries and relationships among anthocerototes (this project) and to provide informative data for the larger community interested in global

phylogenetic questions. A list of 67 characters (available on the project web site) was compiled from previous cladistic analyses (44-46) and more recent data generated by K. Renzaglia and collaborators (6,12,62). In a group where so little is understood about how to define character states it is important to emphasize that these characters will serve as a **baseline for data collection and will be modified substantially as characters are evaluated and character states defined**. It is anticipated that the acquisition of crucial ultrastructural and morphogenetic information combined with a greater appreciation of character definitions will significantly enhance resolution of morphological data.

In addition to accumulating general information on plant morphology, we will conduct intensive studies of key structural features and processes, the most significant of which are described below.

**1. Chloroplasts.** The ultrastructure of chloroplasts separates hornworts from all other land plants and provides important information in circumscription of hornwort genera (47,63). In all but *Megaceros*, the chloroplast is solitary and contains a central pyrenoid. Rubisco localizations in pyrenoids and the lack of grana end membranes are characters shared by hornworts and green algae such as *Coleochaete*. It is generally agreed that pyrenoids were lost and plastid numbers increased in *Megaceros* (64). But, multiple chloroplasts that lack pyrenoids are also seen in species of *Notothylas* and (65) and we have documented the absence of pyrenoids in several putative species of *Phaeoceros*. CO<sub>2</sub> concentrating mechanisms are more active in hornworts with pyrenoids than those without and such a mechanism is undetectable in the liverwort *Marchantia* (66). Additional physiological data (see letter from D. Hanson) coupled with further examination of chloroplast substructure will provide informative taxonomic data. Such studies are necessary to interpret the evolution of these algal-like organelles.

**2. Stomata.** Although stomata are considered to be critical morphological adaptations to life on land and the existence of these structures is viewed as a primary link between hornworts, mosses and tracheophytes (50-52), no modern studies have been published on hornwort stomata. We propose to examine the occurrence, development and structure of stomata in all taxa by examination in the SEM, TEM and fluorescence microscope. Physiological studies in progress in our lab (27), indicate that once open, hornwort stomata do not close and thus do not function as do those of tracheophytes. In addition to continuing studies of stomatal function, we propose to detail variations in substructure and thereby evaluate stomatal evolution within hornworts. The structure and ontogeny of sporophytic stomata will be compared with those of the stoma-like clefts found in the gametophyte of hornworts. Although we hypothesize that these structures are not homologous, others have referred to the mucilage clefts in the gametophyte as true stomata and this issue is of particular importance when comparisons are made with early fossil land plants that possess gametophytic stomata (50,56,67).

**3. Sporogenesis, spores and pseudoelaters.** Spore shape and wall ornamentation, tetrad

organization and pseudoelater characters are widely used in classification of hornwort genera and species (44-46,65). To ascertain variations in spore and pseudoelater differentiation, we will conduct cytomorphogenetic investigations of sporogenesis in all taxa. Non-synchronized spore development in hornworts is viewed as a synapomorphy (52). *Notothylas* is the exception among anthocerotales in that sporogenesis appears to be synchronized (43-46). Our studies of sporogenesis in *Notothylas* suggest that the duration of meristematic activity is dramatically shortened and spore development is not truly synchronized but rather abbreviated in duration. In regards to spore wall ornamentation, little is known of ultrastructural details (68). To test the axiom that wall ornamentation is an informative diagnostic feature of hornwort species and genera, we will observe surface spore wall features with the SEM and correlate these with details of internal organization and development as gleaned from the TEM. To further understand hornwort evolution, comparisons will be made with early land plant spores (see letter from W. Taylor). Elongated single-celled pseudoelaters of *Dendroceros* and *Megaceros* have spiraled wall thickenings while variations in pseudoelater shape and wall thickenings are diagnostic of species (44). Likewise, fluorescence patterns of pseudoelaters (69) are considered taxonomically informative and these will be recorded for all species.

**C. Molecular phylogenetic methods:** We will employ a strategy of collecting multiple molecular data sets will be partitioned so that individual analyses may be completed while allowing extensive tests of

congruence between a number of independent data partitions. The methodologies we will employ are described below.

**1. DNA extraction.** Extraction of total DNAs will be done on fresh material when available. The DNeasy Plant kit (Qiagen) has proven to give high quality DNAs from hornworts where other methods have failed. Furthermore, this kit has been used successfully in J. Duff's lab for DNA extraction and subsequent PCR from herbarium specimens of hornworts up to 55 years old.

**2. Selection of gene sequences and data partitions.** We examined the following candidate genes that could contribute to resolving relationships within hornworts: small and large subunit nuclear ribosomal DNA genes (18S and 26S), nuclear internal transcribed spacer (ITS), *rbcL* (chloroplast), and *nad5*, *nad2* and small subunit rDNA (19S) genes from the mitochondrial genome. Of these gene regions, we have chosen sequences that are useful in reconstructing specific, generic and familial relationships among hornworts. To allow for multiple outgroup options and for comparison of relative rates of sequence evolution, we selected genes with sufficient available sequences from related groups. 19S sequences were found to be too invariant to be useful for resolving within hornwort relationships, while ITS sequences are highly variable and provide resolution of infra-specific relationships but not at the hierarchical levels that are the focus of our proposed research. The lack of introns in the *nad5* gene (mtDNA) in hornworts (39, Duff unpublished data) precludes the use of this gene at different taxonomic levels. *nad2* sequences have been shown to be useful in resolving relationships among other bryophytes (57) and this gene retains two large introns in all hornworts tested. As noted previously, 13 sequences of the first 1900 bp of this gene, including a 1400 bp intron, have been obtained. A large portion of these intron sequences, 1094 bp in the preliminary analysis, can be aligned between genera and exhibit 2.2-6.2% divergence between and up to 2.5% divergence within genera. An additional 57 phylogenetically informative characters (see Fig 2) may be assigned through the limited use of gap-coding (see below) in the 5' intron. To increase the number of characters available for phylogenetic analysis we propose to sequence a large portion of the 5' sequence of *nad2*. Specifically we propose to sequence an additional 500 bp of protein coding sequence and ca. 1600 bp of the intron. Comparisons of two sequences (*Phaeoceros laevis* and *Anthoceros agrestis*) suggest similar divergence can be expected in this region and PCR primers have already been developed and tested. The two introns combined with the exon sequence thus represent ca. 4000 bp of sequence. This along with the 2 kb protein coding sequence we believe will provide the resolution we need to address the goals of this proposal. Figure 5 shows the target sequences and their anticipated resolution based on preliminary data.

**Figure 5.** Characteristics of data sets to be collected and what phylogenetic levels they will resolve. \* denotes combinations of data partitions that will be examined with a total evidence approach. Additional analyses would include the use of different outgroup taxa. Relevant literature and preliminary data suggest the following relationships among the sequences with respect to substitution rates in the target sequences: ***nad2* introns>*rbcL*>18S>*nad2* exon**

Data Partition	Location	Characters	Resolution
Morphology (total combined)	Whole plant	ca. 67	All levels?
18S SSU rDNA	Nucleus	1720 bp	Generic and below
<i>rbcL</i>	Chloroplast	1350 bp	Generic and below
<i>nad2</i> exons	Mitochondrion	1000 bp	Generic and below
<i>nad2</i> intron1	Mitochondrion	1400 bp	Generic and above
<i>nad2</i> intron 2	Mitochondrion	1600 bp	Generic and below
*18S+ <i>rbcL</i> + <i>nad2</i> exon + morphology	N,CP,MT, plant	3137 bp	Generic and above
* <i>rbcL</i> + <i>nad2</i> introns + morphology	N,CP,MT, plant	4227 bp	Generic and below
*All molecular and Morphology	N,CP,MT,plant	6957 bp	All levels

**3. PCR and sequencing.** For amplifying each of the target sequences, we have developed new primers effective in selecting the hornwort sequence from total DNA extractions that may contain cyanobacteria or

algal contaminants. Other aspects of gene amplification, cloning of products, product purification, and DNA sequencing reactions will follow standard protocols. Sequencing will take place on an ABI 310 automated sequencer available in the Department of Biology at the University of Akron. In total, we anticipate generating at least 85 new 18S rDNA [1720 bp] *rbcL* [1370 bp] and *nad2* [ca. 4000 bp] sequences for a total of 603 kb.

**4. Outgroup selection.** Given the conflicting hypotheses on the position of hornworts among land plants (see Background), we will include charophycean green algae (*Nitella*, *Chara*, *Coleochaete*), basal liverworts (*Sphaerocarpos*, *Blasia*, *Haplomitrium*), basal mosses (*Sphagnum*, *Andreaea*) and basal vascular plants (*Huperzia* and *Botrychium*) as outgroup members. To ensure accuracy of plant identification and quality of sequence data, we will generate new data on these organisms. Likewise, morphological studies will be conducted on the same samples. Heretofore, analyses of the *rbcL* and 18S sequences have show that any combination of outgroup taxa has no effect on the topology of the hornwort clade. However, no *nad2* sequences exist for charophycean algae and thus it is crucial to sample these plants. Intron sequences of *nad2* are more difficult to root as only the first intron of hornworts exhibits homology with other plant groups (ca. 450 bp of alignable sequence with liverworts) and the second intron apparently is found only in hornworts. When analyzing *nad2* intron sequences alone we will produce unrooted trees.

**5. Sequence alignment.** Alignment of all sequences will be done visually employing computer programs SeqApp (70) and VectorNTI (InforMax). J. Duff has extensive experience aligning these genes in the context of basal land plants (36,71). Alignment of *rbcL* and *nad2* exons is trivial as they may be unambiguously aligned among all hornworts and outgroups. Length variable regions in the 18S gene are generally limited to a few termini of helices. For our analyses these sites will be excluded. The introns of the *nad2* gene present greater challenges because only a portion of the first intron may be aligned to a single outgroup, the liverworts (57), as mosses and vascular plants are lacking this sequence. Furthermore, the second intron is found only in hornworts. It is unknown if any charophycean alga have the same introns. Within the hornworts the first intron, 1094 bp may be unambiguously aligned among the 12 sequences gathered to date. In addition to these aligned sequences there are numerous indels of 1-25 bp in length. Alignment gaps due to indels do not constitute missing data, as they are typically treated, but are the result of changes in ancestral sequences (72). How these gaps should be coded, though, is far from clear (72-75). As described in the preliminary data, the 1094 bp of unambiguously aligned sequence will be analyzed separately, eliminating all regions involved in length variation. This clearly eliminates much useful information contained in the aligned indels of the majority of the taxa hence we will analyze sequence with gaps included, treating gaps first as missing data as has been done with this gene (57). We will also explore utilizing the 57 identified type I indels (76) that may contain phylogenetic signal and for which alignment gaps may be aligned by the criteria outlined by Golenberg et al. (76). These indels, representing small duplications of adjacent sequence, will be treated as fifth character states and coded as binary characters at the end of the sequence data matrix. Sites not fitting these conservative criteria will be eliminated from subsequent analyses.

Presently there are only 10 relevant hornwort sequences deposited in Genbank. For the **majority** of these sequences **we suspect errors** in either the sequence itself or species identifications. Hence, we propose to use only hornwort and outgroup sequences generated as part of the proposed research for our molecular phylogenetic analyses. We will deposit all sequences into Genbank as well as provide alignments, including all subsets with assumption sets for each analysis, of all data sets on our project web site hosted at the University of Akron: (<http://www.uakron.edu/biology/hornworts/hornworts.html>).

**6. Phylogenetic Inference.** Both morphological and molecular data sets will initially be analyzed separately. Tree constructing algorithms will include distance, maximum parsimony and maximum likelihood as implemented in the software package PAUP\* 4.0 (77). Confidence levels for various clades will be determined using bootstrap (78) and decay analyses (79) implemented with AUTODECAY (80). PAUP\* will also be used to constrain trees to other topologies (e.g., topologies resulting from data partitions not included in the combined analyses due to significant heterogeneity between data sets) thus allowing determination of the costs (in steps) relative to the most-parsimonious solution. Morphological

character evolution will be examined by employing the program MacClade (81). **Molecular Data Analyses:** Transition/transversion ratios will be calculated and reasonable unequal weighting schemes employed. Furthermore third base positions in *rbcl* and protein coding portions of *nad2* will be examined and either down weighted relative to first and second positions or eliminated altogether. Because we are examining relationships at multiple levels different weighting schemes may be required at different levels. For example, *rbcl* third base positions are saturated (36) when examining the deepest nodes and thus some form of weighting or elimination is appropriate. Likewise, analyses aimed at examining interspecific and subgeneric relationships would benefit from inclusion of third base positions. Similar tradeoffs in coding and weighting schemes apply to the *nad2* intron sequences as discussed above. **Morphological Data Analyses:** The fact that morphological characters have produced highly conflicting phylogenetic hypotheses in the past indicates that some characters utilized in taxonomic studies of hornworts are not appropriate for phylogenetic reconstruction. At this time we cannot say whether these incongruences are the result of convergent and parallel evolution of particular features, the result of our ignorance of character homology, or both. We anticipate that our ultrastructural, functional and developmental investigations will enable us to better assess the homology of morphological characters. We will initially develop four combinations of the data (see Figure 3) that will allow comparisons of characters from different life stages (gametophyte vs. sporophyte) and tissue types (vegetative vs. reproductive). With our revised morphological data sets, phylogenetic analyses will be conducted on each partition and combined and the resultant trees compared to the results obtained from molecular data.

**Combined Analyses:** The assembly of a data set that includes multiple DNA loci and morphological characters designed to examine questions at different taxonomic levels necessitates a multifaceted approach to data analyses. We will approach combined analyses using 1) total evidence and 2) the conditional-combination approach, and we will compare results of these two analyses. Maximum parsimony will be the primary method as the size of the data set and multiple data types will limit the use of ML for combined analyses **1) Total Evidence Approach:** Arguments that favor combination of all data theorize that the differences in signal from different partitions in the data set (e.g. due to convergent evolution or differences in evolutionary rate) can only be detected by combined analyses (28-30,82,83) Thus, we will conduct analyses that combine all data sets. **2) Conditional-Combination Approach:** We will also follow the conditional-combination approach (Figure 3, 54,84,85) and we will compare those results with the total evidence approach. Prior to combining data sets, congruence, especially non-coding and coding sequences, will be assessed using the partition-homogeneity test (ILD of 86) and the Templeton's Significantly Less Parsimonious Test (SLP<sub>T</sub>; 58,87). If no significant differences are observed the data sets will be combined and a second round of analyses conducted. If significant incongruencies are found, data sets will be reexamined in light of possible effects of transition/transversion ratios, base composition bias, variations in evolutionary rates among lineages and among sites to identify sources of heterogeneity. If significant heterogeneity still exists and cannot be addressed by differential weighting schemes, deletion of problematic taxa or other methods, then each phylogeny may have to be considered separately. Combined data sets will be further analyzed with methods used for individual data partitions.

**E. Hornwort Classification:** We will provide a rigorous, strictly cladistic classification for the hornworts. We acknowledge that controversy exists as to the applicability of the Linnaean system, at least at the higher levels, but for the sake of expediency, we will classify hornworts according to traditional ranks (88-90). We will also give phylogenetic diagnoses of hornwort taxa, so that their applicability under a future Phylocode will be clear.

**F. Biogeography and biodiversity: hornworts in spatial and temporal perspective:** Similar to the mosses and liverworts, hornwort species exhibit unusual distribution patterns (91,92). Many species are described as cosmopolitan (found on all continents except Antarctica) while others show extremely limited ranges (42). Because of the presence of both pockets of high endemism and widespread species, hornworts present opportunities to investigate many fundamental issues relating to biogeography and speciation. Presently, we seek to identify general worldwide biogeographic patterns and examine specific biogeographic hypotheses involving the Australasian landmasses. This approach of examining biogeographic patterns at multiple levels will allow us to **address the following questions:** Are regions of

high endemism as determined by morphology also characterized by higher levels of genetic diversity? Do species that exhibit cosmopolitan distributions show evidence of widespread gene flow thus reflecting extensive long-range dispersal? Is the high diversity of New Zealand hornworts ancient or recent? Do biogeographic patterns exhibited by hornworts reflect patterns observed in other bryophytes, vascular plants or other organisms?

Our phylogenetic analyses will provide a framework wherein we can investigate biogeographic patterns in the hornworts. Shaw (91) examined bryophyte species complexes from a phylogeographic perspective but few attempts have been made to examine bryophyte biogeographic patterns at higher taxonomic levels in conjunction with phylogenetic analyses (31). Hypotheses have suggested periods of rapid bryophyte diversification that correspond to large scale global crises associated with the end of the Permian (93). Wheeler identified an unresolved polytomy in a relatively advanced clade of the Marchantiales that potentially corresponds to the same period of time. Our preliminary data suggest that the main lineages of hornworts (*Anthoceros*, *Phaeoceros/Notothylas*, and *Dendroceros/Megaceros*) were established early in land plant history but that other periods of rapid diversification may have occurred. With a well-resolved phylogeny we will be able to look for common phylogenetic patterns of diversification that may be correlated with a variety of periods of the Earth's history.

**1. Worldwide patterns of distribution:** To gain a greater appreciation for diversification, we propose to conduct dispersal-vicariance analyses (94) of hornworts collected from all continents except Antarctica. Owing to the antiquity of this group, one might presume that patterns of biogeographical distribution among hornworts would reflect vicariance due to the break-up of the supercontinent Pangaea and later Gondwanaland and Laurasia. The divergence observed in *rbcL* and 18S sequences suggests that many of the modern genera diversified at or before the breakup of the supercontinent Pangaea (>250 mya). By comparison, estimations of divergence times in the Lycopodiaceae using *rbcL* sequences suggest that the extant genera are over 250 mya (95). Assuming similar rates of sequence evolution in hornworts, the observed sequence divergence between genera would suggest they are of similar age. Unfortunately, direct evaluation of divergence times among hornwort genera is not possible because of the difficulty in establishing minimum and maximum divergence times from paleontological evidence, estimates that are possible for the lycopods. Nevertheless, the hornwort data collected thus far are not inconsistent with deep divergences among extant genera. By examining species found on all landmasses, we will be able to test (see methods below) whether patterns of diversity reflect vicariant events.

**2. Australasian patterns of distribution:** On a more limited geographic scale we can provide a more rigorous test of biogeographic and biodiversity patterns among hornworts. We have already obtained material from a large number of taxa from Australasia. The Australasian landmasses consist of Australia (including Lord Howe Island, Norfolk Island and Macquarie Island), New Zealand (including Auckland Islands, Campbell Island, Chatham Island, Kermadec Islands and Bounty Islands), New Caledonia (including Loyalty Islands), Vanuatu (formerly New Hebrides), Solomon Islands (including Santa Cruz Islands), Bougainville, New Britain, and Papua New Guinea (96). These locations, most of which have hornworts, have been of particular interest to biogeographers because of the generally accepted patterns of continental evolution of the region (97,98), although alternative vicariance models have been obtained from molecular phylogenetic studies (99,100). A variety of studies (99,101-106) focusing on this region have implicated both vicariance events and long-distance dispersal in explaining the current biogeographic patterns of a number of plant families. But, despite the presence of a generally accepted geological vicariance model for the austral region (NZ(SA(AUST)), recent studies applying molecular phylogenetic data to biogeographic questions in taxon distributed over this region have recognized dispersal as being the causal agent of the observed patterns of biotic distributions (107-110). In addition to Australia, only Tasmania has yielded definitive evidence of an ancient Gondwana flora reflecting a vicariant biogeographic pattern (111). Some uncertainties remain with respect to the geological histories of several locations included in most biogeographic studies of the area. These include whether New Caledonia, New Zealand, New Guinea, and Chatham Islands have remained emergent (and thus available for colonization) since their initial isolation from their continental relatives (112). If they have, then vicariant events may have played a role in the current distribution of the flora and fauna of these places.

The presence of multiple species from several genera on each of the Australasian landmasses presents an opportunity to examine and compare biogeographical patterns of hornworts with those of other paleoaustral plant taxa for which molecular data have been obtained. Examining member of Megaceros, which are particularly well represented in Australasia, will accomplish this and thus our analyses will focus on deciphering the patterns of diversification of this genus. *Dendroceros* and *Phaeoceros* will also be investigated. For each of these groups of taxa various biogeographical analyses will be applied to the phylogenetic trees. In particular we will conduct dispersal-vicariance analyses using the computer package DIVA as described by Ronquist (95,114). A second method, weighted ancestral areas analysis (114) will be used and the results of these two analyses compared for congruence using the model of *Nothofagus* (100,111).

## VI. Timetable and Future Goals

The large number of collections, morphological observations and DNA sequences makes the collaboration of the two PIs in this project vital to the success of the program. Coordination of molecular studies and generation of sequence data primarily will take place in the lab of J. Duff (U. Akron) while morphological investigations will be coordinated and largely conducted by K. Renzaglia's lab team (SIU). Students will be expected to visit both laboratories (see Budget Justification) and to gain proficiency in a wide range of protocols, and methods of data collection and analysis. Morphological investigations will be ongoing and the *18S* and *rbcL* genes will be a first priority in line with our initial goal to define broad relationships among the hornworts. An emphasis in Year 1 will be to acquire most of the specimens from around the world and process them for analyses. A large portion of this work will involve collection from the Australasian region by Chris Cargill and Jeff Duckett. Year 2 will see a continuation of sample collection and processing and the initiation of sequencing of the entire *nad2* gene. At this time, data sets for the *18S*, *rbcL* and morphological data for a large number of taxa will be complete, allowing preliminary analyses to be conducted, which will inform decisions on additional sampling and/or morphological character examinations. In Year 3, we expect to complete sequencing of the *18S* and *nad2* sequences from those samples for which infrageneric relationships are of interest. We expect that early publications will result from *rbcL* sequences and morphological investigations. Subsequent papers will follow in areas outlined in Figure 3.

The described research will enable us to achieve our goals while providing a qualitative step toward establishing a **long term research program** aimed at 1) continued evaluation of hypotheses of adaptive evolution within the group and placing morphological innovations diagnostic of hornworts into the broader context of land plant evolution, 2) providing an exhaustive taxonomic treatment of the entire group worldwide, 3) integrating our data and insights into complementary projected aimed at elucidating patterns of macroevolution in green plants, 4) placing biogeographical patterns in specific regions of the world observed in hornworts in context with other bryophyte, vascular plants and animals, and 5) exploring bryophyte morphological and genetic complexity, including the existence of cryptic speciation (91), through intensive examination of cosmopolitan species such as *P. laevis* and *A. punctatus*.

## VII. Intellectual Merit and Broader Impact

Rare among the extant groups of eukaryotes, the hornworts represent a living historical record of as many as 500 (-700) million years of evolutionary adaptation. The available studies, e.g., on the cell cycle and sperm structure, have identified cellular complexity that is unique to these plants. Similarly, the hornworts exhibit general architectural features of the gametophyte and sporophyte, as well as strategies for survival on dry land that are unparalleled in other eukaryotes. Our three focus areas (molecular, morphological and biogeographical) center around questions pertinent to both hornwort systematics and more global issues relating to biological diversification and biocomplexity. In spite of their evolutionary importance, the hornworts are the only major group of land plants for which a detailed molecular phylogeny is lacking. Existing classification schemes based on morphology are in great conflict; there is no agreement on generic or familial limits, and the interrelationships among taxa remain obscure. Consequently, it is not possible to identify a single basal exemplar taxon. The shortage of biological information on these plants

also contributes to this lack of phylogenetic resolution. Our proposed research will remedy these inadequacies. Distribution patterns and information on biodiversity will be accumulated for the first time for hornworts collected from multiple geographic regions by an international assemblage of experts on these plants. Morphological studies, coupled with molecular analyses, will provide the necessary data to define taxonomic boundaries and enable accurate estimates of taxonomic diversity. The construction of a worldwide phylogeny of the hornworts will serve as the foundation for a new classification system for the group, and when evaluated in conjunction with distribution areas of the hornwort species will enable a first reconstruction of the biogeographic history of the group.

Reflecting the interests of our large group of collaborators, the proposed work will result in numerous manuscripts in national and international journals, as did the previous NSF grants to K. Renzaglia. To further augment the dissemination and exchange of data and to raise awareness of hornwort biology among the public, we have established a web site dedicated to the project (<http://www.uakron.edu/biology/hornworts/hornworts.html>). This web site is easy to navigate and based on input from educators around the country, has been used extensively as a teaching tool. Through careful networking, we will coordinate our efforts with other laboratories that are conducting similar investigations in other plant groups (e.g., J. Shaw and B. Goffinet, mosses and leafy liverworts; C. Delwiche, charophytes). K. Renzaglia is a co-PI on a collaborative research project (O'Kelly, Mishler, Mandoli, Olmstead, Wolf, Donoghue and Boore) submitted to the Assembling the Tree of Life Program, NSF. This submitted proposal is designed to resolve the primary pattern of evolutionary diversification among green plants (only two hornworts are included) and establish a model for doing so that will be applicable to other groups of organisms with long evolutionary histories. K. Renzaglia is responsible for compiling the morphological data, with emphasis on global ultrastructural features, for key representatives of the embryophyte clades. With emphasis on relationships within hornworts, the proposed hornwort project will complement and strengthen the Tree of Life program (if funded). In any case, we will remain active in research and outreach activities sponsored by the Deep Green Plant Phylogeny Research Coordination Group.

The proposed research will have an impact far beyond the value of the data collected. Both PIs work in research rich environments where significant importance is placed on one-on-one interactions with both undergraduate and graduate students. Our approach to training students is to provide a supportive, stimulating environment that stresses teamwork and provides a strong mentoring network (115-117). K. Renzaglia has a successful record of providing enriching research and professional opportunities for students. This is exemplified by a recent NSF grant that she and Jeff Osborn were awarded to enable minority undergraduates to attend future Botanical Society of America Annual Meetings. Her long-standing commitment to undergraduate education enabled her to attain her present position as director of the undergraduate research program (REACH, Research Enriched Academic Challenge) at SIUC. In this capacity and as an active member of the Center for Systematic Biology, K. Renzaglia will provide a broad base of experiences for students interested in plant systematics. As a new researcher (since Fall, 1999) at a master's granting institution in Ohio, J. Duff has already trained seven undergraduates and one graduate student in his lab. His laboratory and course offerings attract students interested in a wide range of organisms and provide the foundation for students to explore biosystematics and conservation biology. In 2001, J. Duff received the Outstanding Teaching Award from the Honors College in recognition of his commitment to excellence in teaching. He is also a senior person on an educational grant (Herman Muehlstein Foundation) aimed at developing inquiry-based laboratories and providing effective mentoring networks among undergraduates, graduates, and high school students. Rotations between the PIs labs for undergraduate and graduate students participating in the research will provide exposure to the range of techniques and analytical methods that are available to systematists.