A. Introduction: why liverworts?

The early Paleozoic Era was an exciting period in earth's history, marked by the colonization and diversification of terrestrial organisms, including the ancestral lineages of extant embryophytes (Kenrick & Crane 1997). As photosynthetic organisms adapted to the higher CO_2 levels and diminished UV-B screening of emerging terrestrial habitats (Raven 2000), a vast array of morphological innovations were developed (Renzaglia et al. 2000), new patterns of genome structure and gene expression evolved (Kofuji et al. 2003; Nishiyama et al. 2003; Tanabe et al. 2005), and novel symbiotic relationships were established (Pirozynski & Malloch 1975). Data from a variety of sources now indicate that liverworts (Phylum Marchantiophyta) were at the forefront of these ground-breaking developments The liverworts provide a unique window into early land plant genome and morphological evolution. Although the precise sister group to embryophytes is still being debated, outgroup considerations clearly favor the hypothesis that the algal sister group to embryophytes was characterized by a haplobiontic life cycle and zygotic meiosis. The liverworts provide our best record of earliest stages in the evolution of the plant sporophyte (diploid) generation, as well as plesiotypic diversity in the form and development of haploid gametophytes. Inferences from morphology, nucleotide sequences, and genome structure are converging on the hypothesis that liverworts are the earliest divergent branch of embryophytes. The liverwort clade is therefore as old or older than any other land plant lineage and may well be sister to all other extant embryophytes. The depth of the liverwort clade implies unparalleled phylogenetic diversity, but it also appears that some groups of liverworts have undergone relatively recent and extensive radiations at the species-level (Schuster 1979; 1984; Gradstein 1979, 1994, 1997; Forrest et al. in press). The liverworts are thus central to understanding land plant morphologies, and constitute a model group for investigating patterns of phylogenetic diversification (Shaw & Renzaglia 2004).

Project Goals: We propose to reconstruct the Liverwort Tree of Life (LTOL) by combining data from conservative ultrastructural features, gametophyte and sporophyte development and anatomy, organellar genome structure, and multilocus DNA sequencing. In addition to reconstructing the LTOL, a major emphasis is to integrate our efforts with other AToL (and related) projects. Our informatics program extends development of the "Botany Browser" funded through *AToL: Reconstructing the Angiosperm Tree of Life* (D. & P. Soltis et. al.), broadening the Browser's capabilities by incorporating a new "Phylogenetic Diversity Explorer." Our genomic efforts dovetail seamlessly with several other projects focused on plant organellar genome evolution, including the Green Tree of Life AToL Project (O'Kelly et al.; PI: Renzaglia). Our nucleotide sequencing component complement project aimed at resolving the generic level phylogeny of mosses (PIs: Shaw & Goffinet) and hornworts (PI: Renzaglia). Another of our PIs (Crandall-Stotler) currently holds a PEET grant focused on liverworts. The automation and streamlining of sequence manipulation to be utilized in our current work builds on collaborative informatic efforts in place with the AToL project: *Assembling the Fungal Tree of Life* (Lutzoni & Vilgalys et al.).

Liverworts: an early-diverging lineage of land plants – Although the liverwort fossil record is limited, the early occurrence of liverworts is consistent with the hypothesis that they are the sister group to all other land plants. Megafossils date to the Late Devonian (Oostendorp 1987), and microfossils to the middle Ordivician (Edwards et al. 1995; Wellman & Gray 2000; Wellman et al. 2003) or perhaps even the middle Cambrian (Strother & Taylor 2004). Phylogenetic analyses of extant taxa also support liverworts as the sister group to the rest of the embryophytes (Mishler & Churchill 1984 [morphology], Mishler et al. 1994 [nrDNA sequences and morphology], Lewis et al. 1997 [cpDNA sequences], Qiu et al. 1998; Pruchner et al. 2002; Groth-Malonek et al. 2005 [group II mtDNA introns], Steinhauser et al. 1999 [mtDNA editing]). Contradictory data do exist (Hedderson et al. 1996, 1998; Garbary & Renzaglia 1998; Nishiyama & Kato 1999; Renzaglia et al. 2000; Nickrent et al. 2000; Nishiyama et al. 2004), but it is fair to say that the weight of the current evidence strongly favors the liverworts-basal hypothesis. The branching order among the three "bryophyte" groups (mosses, liverworts, hornworts) is beyond the scope of this proposal, and is a focus of the Green Tree of Life Project (O'Kelly et al.). The critical position of liverworts with regard to land plant evolution is uncontested.

A primer of liverwort diversity – There are an estimated 8000 species of liverworts in 377 genera and 74 families (Crandall-Stotler & Stotler 2000). Liverworts occur worldwide in a broad range of environments, but much of the taxonomic diversity is centered in the Southern Hemisphere, especially in cool, moist montane habitats of Latin America, the Indian subcontinent, and Australasia (Schuster 1983).

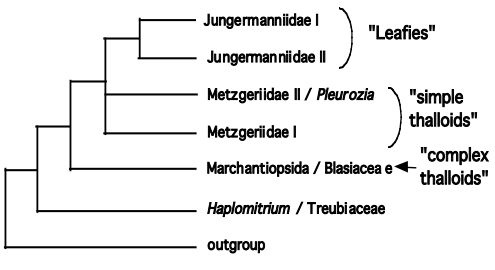
Liverworts are ectohydric and generally adapted to mesic habitats, but there are a few xeric taxa that are desiccation tolerant (Oliver & Bewley 1997) and a few aquatics (Schuster 1981). Liverworts resemble mosses and hornworts in their homosporous, gametophyte-dominant life cycle, and monosporangiate sporophytes, but are unique among the embryophytes in that the sporophytes undergo development totally within the confines of the gametophyte (Crandall-Stotler & Stotler 2000). All recent analyses resolve the liverworts as a single monophyletic group, in contrast to earlier suggestions that they are polyphyletic (Bopp & Capesius 1998; Lewis et al. 1997).

The haploid gametophyte generation is exceptionally diverse in structure. Three basic forms of gametophyte organization occur among the liverworts, commonly referred to as *leafy*, *simple thalloid*, and *complex thalloid* body plans. Traditional systems of liverwort classification largely mirror these three body plans. According to the most recent synthesis (Crandall-Stotler & Stotler 2000), the phylum Marchantiophyta (liverworts) is divided into the classes Marchantiopsida (complex thalloids) and Jungermanniopsida (simple thalloids and leafies). The latter have been further divided into the subclasses Jungermanniidae (leafies) and Metgeriidae (simple thalloids). Hereafter, these groups will be referred to by their descriptive common names. Variation in general body plans is accompanied by significant diversity in fundamental structural characters. In particular, there are three types of antheridial ontogeny and sperm architecture (Garbary et al. 1993), two developmental pathways for gametophytic leaf formation (Crandall-Stotler 1981), four apical cell systems in developing gametophytes (Crandall-Stotler & Stotler 2000), and two or three developmental scenarios in early embryology (Crandall-Stotler 1981). Meiosis can be either monoplastidic or polyplastidic (Renzaglia et al. 1994), and mitotic divisions involve algal-like polar organizers, unknown elsewhere among embryophytes (Steer 1984). This project will investigate the phylogenetic utility of these characters, and interpret their evolutionary transformations in the context of many other types of data.

B. Background – current state of the liverwort tree

Until recently, most molecular phylogenetic analyses that included liverworts were aimed at resolving land plant relationships and the liverworts were represented by only a few exemplar taxa. The first studies with more extensive taxon sampling were directed at the complex thalloids (Wheeler 2000; Boisellier-Dubayle et al. 1997, 2002). More recent molecular work has focused on familial structure (Ahonen 2004, Ahonen et al. 2003; Long et al. 2000; Schill et al. 2004; Wilson et al. 2004; Yatsentyuk et al. 2004), the position of morphologically ambiguous taxa (Stech & Frey 2004; Stech et al. 2000), or interspecific relationships (Fiedorow 2001; Forrest et al. in press; Groth et al. 2002, 2004; Heinrichs et al. 2002a, b; 2004; Kim et al. 2001; Miwa et al. 2003; Pfeiffer 2000; Pfeiffer et al. 2002; Renker et al. 2002; Rycroft et al. 2002; Stech et al. 2002; He-Nygrén & Piippo 2003; Schaumann et al 2003). Crandall-Stotler & Stotler (2000) reconstructed liverwort relationships based on 61 morphological characters and Crandall-Stotler et al. (in press) have reconstructed morphological character evolution in the Metzgeriidae, with a total evidence analysis incorporating data from 8 loci and 65 morphological characters for 50 taxa. Most recently, Crandall-Stotler, Stotler & Mishler (in preparation) have expanded this analysis to include fossil taxa. Papers utilizing more extensive taxon sampling and multilocus DNA sequences are beginning to appear, based mainly on work in the labs of PIs on this proposal: e.g., Davis (2004, Shaw lab) focusing on the leafy liverworts and Forrest & Crandall-Stotler (2004, 2005) focusing on the simple thalloid liverworts. A progress report on liverwort phylogenetics is being presented by L. Forrest & B. Crandall-Stotler (SIU), C. Davis (Duke), J. Heinrich & R. Wilson (Goettingen), and D. Long (Edinburgh), at the 2005 IBC in a “bryophyte” phylogeny symposium organized by J. Shaw.

Figure 1.



Recent molecular analyses of liverwort relationships have suggested some resolution of major clades, but relationships within the major clades are still largely unresolved (Fig. 1). Provisionally, *Haplomitrium* and *Treubia* form a monophyletic group that is sister to all other liverworts (Forrest & Crandall-Stotler (2004; 2005; Crandall-Stotler et al. in press; Stech & Frey 2004). In contrast, *Haplomitrium* (without *Treubia* in the analysis) was resolved sister to the leafies plus simple thalloids by Davis (2004). Our project will resolve this issue. The

complex thalloids plus *Blasia* are sister to the remaining simple thalloids plus leafies (Fig. 1). The simple thalloids are likely paraphyletic (Davis 2004, Forrest & Crandall-Stotler 2004, He-Nygrén et al. 2004), with one group (Metzgeriidae I) sister to a clade comprising the leafies plus another group of simple thalloids (Metzgeriidae II). Our extensive data from diverse character sets will also provide resolution to this issue. Leafy liverworts, excluding *Pleurozia*, form a monophyletic group. In fact, one of the greatest enigmas to come from molecular analyses to-date is the placement of *Pleurozia*, with its leafy gametophytes, in the Metzgeriidae II group (Davis 2004; He-Nygrén et al. 2004, Crandall-Stotler et al. in press). Two major clades are otherwise resolved within the leafies, one including the Porellales and Radulales (Jungermanniidae I), characterized by complicate-bilobed leaves with ventral water sacs, and the other (Jungermanniidae II) including all other leafies. Some taxa "float" outside either clade (e.g., *Ptilidium*) and their placement is currently ambiguous. Although the leafies include at least 80% of all liverwort species, sampling within these clades has been so sparse that virtually nothing is known about familial and ordinal relationships, nor therefore about evolutionary transformations in leafy gametophyte morphology.

C. Data Acquisition

The general research strategy for resolving the liverwort Tree of Life – We will utilize three types of data to resolve phylogenetic relationships: morphology, genome structure, and targeted nucleotide sequencing. By using heterogeneous types of data, we take advantage of conservative characters for reconstructing deep nodes and more labile characters to resolve more "shallow" relationships. Ultrastructural and developmental characters are relatively conservative and mark deep phylogenetic divergences (Crandall-Stotler 1984; Carothers & Rushing 1988; Garbary & Renzaglia 1998; Garbary et al. 1993; Renzaglia & Garbary 2001). Characters pertaining to genome structure have demonstrated utility for resolving deep relationships in seed plants and mosses. Ultrastructural, developmental, and genomic characters are, however, labor- and time-intensive to gather, limiting the number of taxa that can be sampled, and may be invariant among closely related OTUs within major clades. Consequently, these data must be complemented by targeted nucleotide sequencing and general morphological characters to resolve relationships across the whole spectrum of liverworts. Neither morphology (Crandall-Stotler & Stotler 2000, Crandall-Stotler et al. in press), nor molecules independently (Davis 2004; Forrest & Crandall-Stotler 2004, 2005) have resolved liverwort relationships.

Taxon sampling: Three nested data sets

Three data sets will be developed to resolve "backbone" relationships among the 4-6 deepest liverwort clades (Data Set 1), major lineages within these clades (Data Set 2), and among genera and species across the liverworts (Data Set 3). Because of resource limitations and patterns of variation (more or less conservative characters), different sets of characters will be scored for each data set. Each data set will include all the characters scored for the more taxon-inclusive data sets within which they are nested, plus those characters limited to that data set.

Data Set 1:18 exemplar species of the deepest liverwort clades (Table 1)

- whole plastid and mitochondrial genome sequences
- >140 ultrastructure characters (Morphology I)
- all characters scored from Data Sets 2 & 3

Rationale for taxon selection in Data Set 1: Our choice of exemplars (Table 1), while guided by their hypothesized phylogenetic position, is also tempered by the sampling in other projects and the accessibility of adequate material, either as axenic cultures, fresh collections, or samples prepared for microscopic examination. We have also given preference, when possible, to those taxa that are frequently used as model organisms. Ultrastructural, embryological, and whole chloroplast and mitochondrial genome sequence data will be gathered for all exemplars; taxa thought to be early diverging lineages within each clade are targeted in this data set.

Table 1. Exemplars for organellar genome sequencing and ultrastructural studies. cp = chloroplast genome; mt = mitochondrial genome; sm = spermatogenesis data; sp = sporogenesis; pl = placental studies; g = gametophyte meristem ultrastructure; 1 = first priority for data acquisition, this proposal, 2 = second priority for data acquisition, this proposal; ax = axenic cultures available from CS lab. Data available from other studies are indicated with references as follows: a, O'Kelly et al. (Green tree of Life Project); b, Ohyama et al. (1986); c, Wickett (in prep.); d, Qiu (in prep); e, Oda et al. (1992); f, Carothers & Rushing (1990); g, Garbary et al. (1993); h, Renzaglia et al.

(1985); i, Rushing & Carothers (1986); j, Renzaglia et al. (1994); k, Brown & Lemmon (1988); m, Ligrone et al. (1993); n, Carafa et al. (2003); o, Bartholomew-Began (1991).

Exemplar	Lineage represented	Genome Sequences		Ultrastructural data				Specimen source
		cp	mt	sm	sp	pl	g	
Treubia lacunosa	Treubiales, basal lineage	1	1	f	1	n	1	New Zealand
Haplomitrium mnioides	Haplomitriales, basal lineage	a	a	g	a	a	o	ax
Sphaerocarpos texanus	Marchantiopsida, Sphaerocarpiidae	a	a	a	a	a	1	ax
Marchantia polymorpha	Marchantiopsida, Marchantiidae	b	e	g	a	1	1	greenhouse, SIUC
Reboulia hemisphaerica	Marchantiopsida, Marchantiidae	2		2	2	m	2	ax
Blasia pusilla	Metzgeriidae, Blasiales: sister to Marchantiopsida	a	a	g	J	m	1	ax
Aneura pinguis	Metzgeriidae II	c		1	1	m	1	ax
Pallavicinia lyellii	Metzgeriidae I	1		h	k	m	2	ax
Pellia epiphylla	Metzgeriidae I	1	1	g	1	m	1	ax
Pleurozia purpurea	Jungermanniidae, Pleuroziales: sister to Metzgeriidae II	1		1	1	1	1	Britain, western US
Porella navicularis	Jungermanniidae I, basal lineage	1	1	1	1	1	1	ax
Radula obconica	Jungermanniidae I	2		2	2	2	2	ax
Scapania nemorea	Jungermanniidae II	d	d	1	1	1	1	ax
Nowellia curvifolia	Jungermanniidae II	2		2	2	2	2	ax
Bazzania trilobata	Jungermanniidae II	a	a	i	a	a	1	ax
Herbertus aduncus	Jungermanniidae II	2		1	1	1	1	North Carolina
Jungermannia leiantha	Jungermanniidae II	2		2	2	2	2	ax
Calypogeia muelleriana	Jungermanniidae II	2		2	2	1	2	ax

Data Set 2: 100 species: 65 leafies, 20 simple thalloids, 15 complex thalloids

- mitochondrial gene intron presence / structure
- 40 anatomical and developmental characters
- all characters scored for Data Set 3

Rationale for taxon selection in Data Set 2: Here we sample synoptically within the 3-6 deepest clades to resolve relationships at intermediate phylogenetic depths. Everything else being equal, we will target early diverging taxa to represent clades. Other considerations include availability of suitable material for complete morphological characterization, including developmental features wherever possible. We focus our sampling on the leafies, which are most diverse in terms of both species richness and morphological disparity.

Data Set 3: 900 species: 750 leafies, 90 simple thalloids, 60 complex thalloids

- nucleotide sequences for 12 loci
- 38 morphological characters

Rationale for taxon selection in Data Set 3: The goal here is to sample the full range of phylogenetic diversity among liverworts. At least 3/4 of all liverwort species have leafy gametophyte morphologies, yet phylogenetic relationships among leafy genera are least known. We aim to sample multiple species from every genus, recognizing of course that some genera will be difficult to obtain since they are known from only one or a few collections. A few of the leafy families are exceptionally rich in genera and species; for example, the Lepidoziaceae with 27 and the Jungermanniaceae with 36 genera. The Lejeuneaceae, with 93 genera and over 1000 species are truly exceptional (Gradstein 1979; 1994, 1997). This hyperdiverse family is largely tropical and includes many epiphyllic species of lowland and montane tropical rainforests. Our sampling of Lejeuneaceae will be conducted in collaboration with S. R.

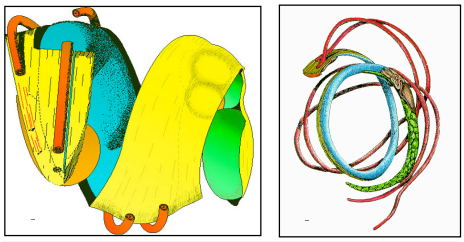
Gradstein and J. Heinrichs (see letter). We use the generic classification of Crandall-Stotler & Stotler (2000) as a framework for our taxon sampling.

Specimen resources: Much of the morphological data and nucleotide sequences to be compiled for Data Set 3 can be extracted from herbarium material. The Field Museum (F) has an outstanding collection of liverworts that can be sampled judiciously, including one of the most extensive samplings of Southern Hemisphere endemics, and the hepatic herbarium at SIUC houses an excellent collection of North American and European taxa. In addition, collaborators from Edinburgh, Helsinki, Goettingen, New Zealand, and western North America have agreed to provide collections (see letters). The Crandall-Stotler lab (SIUC) maintains an extensive axenic culture collection of liverworts (see http://bryophytes.plant.siu.edu/) for ultrastructural, ontogenetic and genomic sequencing studies. One field trip to New Zealand is planned to collect otherwise unavailable species for ultrastructural, ontogenetic and molecular analyses.

Character sampling: morphology, genomics, nucleotide sequences

Morphology I: Ultrastructural characters – Studies at the ultrastructural level have revealed substantial diversity in development and organization, and have contributed critical information in ascertaining the evolution of structures and biological processes in early land plants (Brown & Lemmon 1988, 1990a,b,c; Ligrone et al. 1993; Garbary & Renzaglia 1998; Renzaglia et al. 2000; Renzaglia & Garbary 2001; Shaw & Renzaglia 2004). We will conduct detailed ultrastructural studies on the taxa in Data Set 1, focusing on spermatogenesis, sporogenesis (including meiosis), the sporophyte-gametophyte junction, and the gametophytic growing point (including mitosis). With thorough comparative ultrastructural studies of strategically selected taxa representing nodes of diversification within liverworts, we will be poised to: 1) develop a robust data matrix based on developmental and structural homologies, and 2) analyze the evolution of fundamental cellular features and processes based on robust molecular phylogenies produced in our project. Our data set will complement and strengthen ultrastructural investigations across lands plants and green algae funded by the Green Tree of Life AToL Project (PI: Renzaglia).

Spermatogenesis: The strong phylogenetic signal in data relating to spermatogenesis is well documented, especially within liverworts (Renzaglia & Garbary 2001). Indeed, even before the existence of molecular sequence data on liverworts, male gametogenesis supported a sister group relationship between *Treubia* and *Haplomitrium* and a deep evolutionary separation between these two taxa and the remaining liverworts (Garbary & Renzaglia 1993). Ultrastructural similarities in male gamete development and architecture provided the first compelling evidence for a relationship between *Blasia* and the complex thalloids (Carothers & Duckett 1980; Renzaglia & Duckett 1987a, 1987b; Pass & Renzaglia 1995). Substantial ultrastructural variation among liverworts and the existence of diagnostic characteristics within lineages examined to data point to the importance of expanding examination of male gametogenesis in all major liverwort clades (Renzaglia & Duckett 1991). Based on a working list of 75 characters related to spermatogenesis (Renzaglia & Garbary 2001), we will collect comparative information on the development of the male sex organ, differentiation of spermatogenous tissue, and the ontogeny and architecture of the mature motile cell.



Yellow=Spline; Red=Flagella; Blue=Nucleus

Sporogenesis: Spores were among the most significant innovations that accompanied the transition of green plants from water to land (Graham 1993). Comparative studies of sporogenesis have provided crucial information on the evolution of meiosis and the cellular control of spore wall deposition (Brown et al. 1986; Brown & Lemmon 1988, 1990a). For example, within liverworts, monoplastidic meiosis apparently has been lost several times (Renzaglia et al. 1994). Since the earliest fossil evidence of land plants comes from spores of presumed liverwort affinity, ultrastructural studies will provide important data for comparisons of spores between extinct and extant taxa. Moreover, spore surface ornamentation is widely used in taxonomic treatments of liverworts without consideration of wall development and internal organization. We propose to examine the following processes related to sporogenesis in all targeted taxa: 1) sporocyte differentiation, 2) meiosis and cytokinesis, 3) spore wall differentiation and 4) cytoplasmic maturation. We are currently working with a list of 40 characters related to sporogenesis that are designed to include spore features of both living and fossil plants.

Placenta: The sporophyte, a synapomorphy of embryophytes, evolved in bryophytes as a simple generation comprising a foot, seta, and solitary capsule (Shaw & Renzaglia 2004). The liverwort sporophyte is matrotrophic throughout its life span, and the sporophyte-gametophyte junction, or placenta, is the site of nutrient exchange between generations (Graham & Wilcox 2000). The placenta exhibits a number of cellular features that are phylogenetically informative and only visible in the transmission electron microscope (Frey et al. 2001; Ligrone et al. 1993). From our on-going comparative studies of early land plant placentas, we have identified 15 informative characters related to the development and ultrastructure of the foot, surrounding gametophyte, and intervening placental matrix.

Generative apex of the gametophyte: A ubiquitous feature of the growing region in bryophytes is the existence of a single generative cell with a defined geometry (Crandall-Stotler 1981, 1984; Shaw & Renzaglia 2004). This apical cell segments in a precise pattern, producing derivatives that form repeating modules of leaf and stem, or thallus. Mitosis is concentrated in this region and cell differentiation can be followed from apex toward the thallus or shoot base. Our comparative ultrastructural studies of liverwort apices will provide critical data on cell division, which is only superficially known in liverworts and is essential for understanding relationships with algal and bryophyte outgroups (Steer 1984; Brown & Lemmon 1990b). Equally significant is the detailed information on development and ultrastructure that these studies will generate on cellular entities such as oil bodies, chloroplasts, and plasmodesmata. Ten characters will be scored based the ultrastructure of the apical cell and derivatives.

Morphology II: Development – A combination of anatomical and ontogenetic studies will be undertaken for the 100-taxon Data Set 2. These investigations will focus on gathering four classes of data from axenic culturing or with microtome sectioning methods: (1) 8 characters from spore germination patterns and sporeling characters (from cultures), (2) 7 characters from meristem organization in mature gametophytes (serial sectioning), (3) 11 characters from gametophyte stem, leaf, and gametangial anatomy (serial sectioning), and (4) 14 characters from embryology/sporophyte anatomy (serial sectioning).

Spore germination and sporeling patterns: Sporeling patterns, which include features of spore germination, protonemal form, and juvenile plant morphology, have long been considered informative for resolving deep relationships (e.g., Fulford 1956; Nehira 1983; Bartholomew-Began 1985, 1996). The juvenile growth phase often possesses a different apical cell geometry, segmentation type, symmetry, and leaf morphology from the adult plant (Crandall 1969). These morphogenetic patterns that transform the algal-like protonema to a shoot or thallus are highly conserved and will provide a significant group of phylogenetically informative characters (Bartholomew-Began & Crandall-Stotler 1994). Spore cultures will be initiated in the Crandall-Stotler lab, using established techniques.

Meristem organization and gametophyte anatomy: Although some data can be extracted from previous studies (e.g., Crandall 1969; Hébant 1977; Renzaglia 1982), there are major gaps in our knowledge. Either freshly fixed or restored herbarium samples will be prepared for paraffin sectioning using standard protocols. Integration of data on meristem organization derived at this level with the ultrastructural data will allow us to maximize accuracy of homology assessments.

Embryology and sporophyte anatomy: The few studies of early embryology that have been published (e.g., Kienitz-Gerloff 1874; Hy 1884; Schertler 1979) suggest that embryological development in liverworts usually begins with the formation of 2 cells by a transverse division of the zygote. The number of additional transverse divisions that occur prior to multi-dimensional growth and the developmental fate of these two cells, however, is variable and likely lineage-dependent (Campbell 1954; Schuster 1966; Ligrone et al. 1993). Our studies of early embryology will focus on the 18-taxon Data Set 1. Studies of later stages in embryology, form and development of gametophyte-derived investing structures, sporophyte organogenesis, and mature sporophyte anatomy, will include the additional 80 taxa of Data Set 2.

Morphology III: Anatomy and morphology. Generic sampling - Liverworts are phenomenally diverse; gametophytes range from erect and leafy with internally differentiated conducting tissues to flattened thalli with mesophyll-like air chambers and dorsal pores, while sporophyte structure varies from elongate sporangia bearing spores and elaters, subtended by a massive seta and foot, to gametophyte-embedded sporangia containing only spores. This heterogeneity has led to numerous, contrasting hypotheses of character evolution in hepatics and therefore early land plants (Bower 1908; Cavers 1911; Evans 1939;

Schuster 1972, 1984). The development of a comprehensive, morphological database, comprising light microscope and SEM characters for the 900 taxa of Data Set 3, is a major component of this proposal.

Thirty-eight characters, applicable across the diversity of the lineages, will be scored from each DNA voucher specimen, supplemented with additional herbarium specimens as necessary. The characters included in this list have been phylogenetically informative at the distal nodes in other analyses (Crandall-Stotler & Stotler 2000; Crandall-Stotler et al. in press). Well-documented liverwort megafossils from the Paleozoic and Mesozoic also will be included, with data compiled mostly from primary paleobotanical publications and/or illustrations. This approach has proven successful in resolving the position of selected megafossil taxa in phylogenetic studies of the Metzgeriidae (Crandall-Stotler et al. in preparation).

Organellar genomics: General considerations – Genomic structural characters, e.g., changes of gene order, gene duplication and loss, intron gain and loss, change of intron structure and splicing mode, and insertion of transposons, have played an important role in phylogenetic reconstruction over the last two decades (Jansen & Palmer 1987; Manhart & Palmer 1990; Doyle et al. 1992; Raubeson & Jansen 1992; Sankoff et al. 1992; Stein et al. 1992; Boore et al. 1995, 1998; Qiu et al. 1998; Nikaido et al. 1999; Lee & Manhart 2002; Dombrovska & Qiu 2004; Kelch et al. 2004; Qiu & Palmer 2004; Roy 2005; Goffinet et al. in press). Although transformations in genomic characters can be homoplasious (Martin et al. 1998; Mindell et al. 1998; Dowton & Austin 1999; Hillis 1999; Hickerson & Cunningham 2000), the level of homoplasy is typically low (Kelch et al. 2004). Like any data used by systematists, however, genomic structural characters come with their own baggage. A disadvantage is that these characters are far less abundant than nucleotide variations. This potential limitation is, however, being overcome as more genomes are reconstructed (e.g., Wolf et al. in press; Green Tree of Life Project – PI: O'Kelly). Confidence levels for character state transformations can be incorporated into the process of making phylogenetic inferences (Qiu et al. 1998). Ideally, these characters should be analyzed in combination with sequence and morphological data (de Queiroz et al. 1995; Qiu & Palmer 1999). Three sets of genomic characters can potentially contribute to resolving relationships among major lineages of liverworts. (1) intron structure and position within mtDNA genomes, (2) plastid DNA gene order and intron structure, (3) gene losses and horizontal transfers between genomes. In addition to genome structural characters, our plastid genome sequences will permit phylogenetic analyses based on the nucleotide sequences themselves. Sequence-based reconstructions sometimes conflict with inferences gained from structural characters (Nishiyama et al. 2004; Wolf et al. in press), and additional data are needed to reconcile these patterns.

Organellar genomics I: whole plastid genome sequences – Major genomic reorganizations and transfers from the cytoplasmic to the nuclear genome characterize particular lineages of land plants (e.g., Turmel et al. 1999; Adams et al. 2002; Martin et al. 2002; Adams & Palmer 2003; Hackett et al. 2004; Kelch et al. 2004). The chloroplast genome of *M. polymorpha* has 19 of the 21 group II introns that were present in the common ancestor of land plants (Ohyama et al. 1986; Turmel et al. 2002) and have been vertically inherited in streptophytes since (Qiu and Dombrovska, submitted). There is also one group I intron in the *M. polymorpha* chloroplast *trnL* gene that extends back to cyanobacteria (Besendahl et al. 2000). Two of the group II introns present in Charophyceae have been lost in *Marchantia*. Other structural changes in the genome include losses of genes following transfer to the nucleus (Martin et al. 2002) or more rarely to the mitochondrion (Nakazano & Hirai 1993; Zheng et al. 1997), and gene rearrangements. Such transformations in genome structure are likely to have occurred during the long history of liverwort diversification. The loss of the *rpo*A gene and inversion of 72kb of the large single copy unit of the cp genome described for *Physcomitrella* (Suguira et al. 2003) have recently been shown to diagnose major lineages of mosses (Sugita et al. 2004; Goffinet et al. in press). Our sampling will strengthen the basis for understanding chloroplast genome evolution in green plants (Korpelainen 2004; Martin et al. 1998; Timmis et al. 2004).

cpDNA genome methods: We will sequence the chloroplast genome of 11 taxa to include representatives of all major lineages of liverworts (Table 1), complementing efforts by the Green Tree of Life Project (see letters from Mishler & Dandoli). Chloroplast DNA will be extracted using routine protocols (e.g., Qiagen Dneasy Plant kit). Long-Range PCR (L-R PCR) using the Takara amplification kit will be utilized to isolate specific plastid DNA regions, as accomplished for the hornwort *Anthoceros formosae* (Kugita et al. 2003) and the flowering plant *Calycanthus fertilis* (Goremykin et al. 2003). Amplified regions

will then be fragmented, subcloned (Topo Invitrogen shotgun cloning kit), and prepared for sequence analysis using the ABI Big Dye Terminator kit. The L-R PCR method has the advantage of requiring small amounts of DNA. DNA extracts used for routine amplification of targeted loci may suffice both quantitatively and qualitatively to yield large fragments. We have successfully amplified a 14kb fragment spanning the *rpl*14 and *rbc*L genes for one of the targeted taxa, *Aneura pinguis*. Primers used in L-R PCR are designed based on the *Marchantia* genome. Colinearity of genomes is essential to the L-R PCR approach (Jansen et al. in press). Should amplification of targeted regions fail for a particular taxon, we will rely on the fosmid approach, which we are currently using in collaboration with de Pamphilis to isolate the genome of the parasitic liverwort *Cryptothallus mirabilis* (N. Wickett [Goffinet lab]). Sequence annotation will be done using DOGMA (Wyman et al. 2004) complemented by Blast searches should loci correspond to Open reading frames which are not included in DOGMA. The phylogenetic significance of (not necessarily uncommon: Huang et al. 2002) structural transformations will be further evaluated by screening taxa of Data Set 2 using targeted PCR (Goffinet et al. in press).

Organellar genomics II: Mitochondrial gene intron structure – The mitochondrial genome of *Marchantia polymorpha* has 25 group II and seven group I introns (Oda et al. 1992). A pseudogene of *nad*7 occurs in this genome. Most of the mtDNA introns of *M. polymorpha* appear to be uniquely present in liverworts and have not been found at the same gene positions in charophytes or other land plants (Qiu & Dombrovska, submitted). The 100 species comprising data set 2 will be completely or partially sequenced to examine the distribution of all 32 introns in 17 genes known so far only in *M. polymorpha*. Because most of these introns are uniquely present in this species, they must have originated via transposition in a common ancestor or during the evolution of liverworts. Identifying the phylogenetic points of gain of these introns will yield high-value phylogenetic information, particularly if several of them were gained at the same time. Additionally, intron losses are likely. In the mitochondrial large subunit rRNA gene we sequenced for reconstructing land plant phylogeny, a phylogenetically informative intron loss occurs in leafy liverworts. We plan to sequence the full length of 17 intron-containing genes from 40 species to obtain both exon and intron sequences. For the remaining 60 species, all known exon-intron boundaries will be sequenced to determine intron presence/absence. This objective integrates into our ongoing effort to develop a quantitative method to evaluate competing hypotheses of intron gains and losses (G. Estabrook & Y. Qiu, in progress).

In addition, the mitochondrial genome from three liverworts will be completely sequenced to achieve the following goals. First, these data together with those currently being gathered in Qiu's lab (*Scapania nemorosa* and four other basal land plants) and the Green Tree of Life Project (see http://ucjeps.berkeley.edu/TreeofLife/data_table.php, and letters from Wolf & Mishler) and those in the literature (Knoop 2004), will provide an overview of mitochondrial genome evolution and structural stability throughout streptophytes. Second, whole genome sequences will facilitate primer design for the intron survey and targeted gene sequencing components of the project. Finally, these data will be combined with other available mitochondrial genome sequences in a whole-genome phylogenetic analysis to provide an important comparison with inferences based on plastid genomes and targeted gene sequencing. This comparison is important because lineage-specific base composition bias, RNA editing, and substitution rate heterogeneity in charophytes, liverworts, and other basal land plants can influence performance of phylogenetic methods.

mtDNA genome methods: For isolating mitochondrial DNA, we will continue to use the CopyControl™ Fosmid Library Production Kit (EPICENTRE, Madison, WI), which we are using in our project on basal land plant mitochondrial genomes. Different methods have been used to isolate organellar DNA for whole genome sequencing (Jansen et al. in press), but the fosmid approach has two advantages in our case: the requirement of much less tissue (2-5 g versus 20-100 g by other methods), and easy sequence assembly for rearranged genomes, which may occur in liverworts. The degree of mitochondrial genome rearrangement is unknown in liverworts. The mitochondrial genomes of *Chaetosphaeridium globosum* and *Chara vulgaris* (Turmel et al. 2002, 2003) are colinear to that of *M. polymorpha* in many parts, but between these three species and angiosperms, there is very little gene order conservation (Knoop 2004). The uncertainty of genome colinearity also prevents long-range PCR from being a viable approach for isolating genomic fragments, as we propose to do for plastid sequencing described above. Once sequenced, annotation of the genome can now be greatly facilitated by the DOGMA software developed by Wyman et al. (2004). Our collaboration includes several experts on

organellar genome evolution and they will provide guidance on genome annotation and analysis (see the support letters from Boore, de Pamphilis, Jansen, Knoop, Lemieux, Palmer, Turmel, Wolf).

Nucleotide sequencing: multilocus sampling of 900 species – The following loci have been utilized by the PIs on this project in previous studies to resolve phylogenetic relationships in the liverworts: nuclear – 18S and 26S rDNA; plastid – *atp*B, *psb*A, *psb*T, *rbc*L, *rps*4, *trn*G, *trn*L; mitochondrial – *nad*1, *nad*5, *trn*S. Although not all have been utilized in a single study, our results provide strong empirical evidence that these loci are sufficient to resolve generic relationships. Our targeted sequencing study will have the advantages of both extensive character and taxon sampling (Hardeep et al. 2003; Pollock et al. 2002; Zwickl & Hillis 2002). Previous work on liverworts in laboratories of the PIs resulted in minimal incongruence among loci. Additional mitochondrial and plastid loci will become available because of insights gained from our genomic studies, and these will be utilized if necessary. Sequencing of the *Physcomitrella* nuclear genome, presently in progress, is likely to facilitate additional single-copy nuclear loci that can be used in our analyses. The loci we propose to use include a range of substitution rates from more to less conserved, and together they are effective for "shallow" as well as deeper relationships. In a recent liverwort study (Forrest & Crandall-Stotler 2005), the following order was observed with regard to nucleotide variability in eight of these loci: *trn*L>*rps*4>*rbc*L>*nad*5>*atp*B>*psb*A>nrLSU>nrSSU. The deepest "backbone" nodes will be resolved by utilizing sequence data in combination with genomic and anatomical / morphological characters. Our published and in-press preliminary results indicate that this combination of loci resolves both deep and shallower nodes, generally with strong support.

D. Archiving voucher information

In contrast to the case with many better-known groups of organisms, the problem of assuring accurate specimen identification is significant in liverworts. Many specimens in herbaria are misidentified and often contain mixtures of two or more taxa so it is not reasonable to assume that identifications on herbarium labels are correct. J. Engel, M. von Konrat, and R. Stotler, will verify the identification of all collections used for morphological and molecular analyses. This process will be critical to the project and will involve contributions from international specialists for particular taxonomic groups. Voucher specimens will be housed in the herbarium at the Field Museum. The Field Museum bryophyte collection consists of more than 180,000 specimens, including over 2,200 types. Voucher collection information will be stored on KE EMu, a collection management system based at the Field Museum. Data will be accessible on-line (http://emuweb.fieldmuseum.org/botany/Query.php). In addition, these data will be mirrored into a DiGIR provider node (a web service designed for distributed collection data) to further facilitate data access through the Botany Browser (see below).

E. Phylogenetic analysis

Character analysis – Phylogenetic reconstructions are only as good as character analyses are thorough. Homology can be defined on a number of different levels (Patterson 1988; De Pinna 1991); nevertheless, homology assessments are crucial, and the difficulties associated with these assessments vary among character types and data sets. We have considered homologies among ultrastructural characters in a series of previous publications (see especially Renzaglia & Duckett 1991, Shaw & Renzaglia 2004). Anatomical / morphological characters will be evaluated to determine if discrete states can be unambiguously identified and if different characters vary independently. Non-independence can reflect developmental and/or genetic correlations, or multiple characters each tracking the same underlying genealogical history. Developmental criteria are critical to tease these factors apart. Likely homologies among structural genomic features will be assessed by positional criteria, complexity of the transformations, and through the general principle of reciprocal illumination by comparisons among characters. Preliminary phylogenetic results will undoubtedly suggest character re-evaluations in some cases. Homology assessments for nucleotide sequences depend on the level of analysis: ambiguous regions in global analyses can be re-evaluated for more local analyses among closely related taxa.

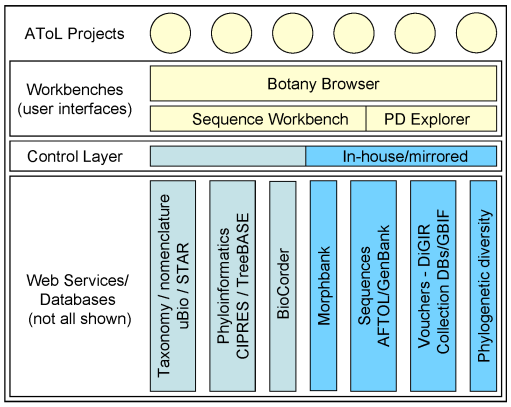
Phylogeny reconstruction – We will compile several data sets that vary from the taxon-extensive nucleotide plus morphology Data Set 3, to the more taxon-limited Data Set 1 scored for conservative ultrastructural and genomic characters. Our challenges therefore include dealing with different sorts of characters scored for a single data set, combining information from different data sets with nested

subsets of taxa, and analyzing very large data sets. Our general approach to character heterogeneity will be exploratory – all else being equal we take a total evidence approach, although we will also conduct separate analyses of different data partitions, examine well-supported conflicts, and pursue analyses of various character sets. We will also address the issue of integrating global versus local analyses with a variety of approaches. These include "backbone" analyses in which deep clades are identified (Data Set 1) based on our most conservative characters, and the more taxon-extensive analyses are constrained to preserve well-supported relationships. A complementary approach is so-called compartmentalization (Mishler 1994), in which global analyses are conducted to identify well-supported clades, and additional analyses of these clades are conducted with all available data (including nucleotide sites that were previously excluded in more global analyses because of homology ambiguities). Final global analyses are then conducted either by constraining relationships according to independent backbone results, or by representing each compartment (clade) by a new "archetypal" OTU. These archetypes are formulated by reconstructing ancestral states for all characters, in effect reconstructing the hypothetical ancestor for each compartment/local clade.

We will utilize a variety of optimality criiteria in our analyses of phylogenetic data: weighted and unweighted parsimony, ML, Bayesian. The analysis of very large data sets is a challenge (Soltis & Soltis 2000) that is currently being addressed by the CIPRes group and we will have access to innovative new approaches to data analysis as they develop (see letter from Lewis). The Bioinformatics Facility in the Biotechnology Center at UCONN Storrs has a 32-processor (2.3 GHz, 64-bit) Apple Xserve G5 computer cluster available for performing computer-intensive phylogenetic analyses (e.g. maximum likelihood bootstrap or Bayesian analyses). The Mac G5 Unix version of PAUP* 4b10 and the MPI-parallel version of MrBayes are available for use on this cluster. The cluster is equipped with a BioTeam iNquiry web interface, which facilitates initiating, tracking the progress of analyses, and managing output.

F. Bioinformatics

Web services federated framework for botanical AToLs – A major goal of our bioinformatics research is integration with other funded and proposed projects, as well as with developing efforts in the new NSF-funded National Evolutionary Synthesis Center (NESCent; see letter from Cunningham). Following the November 2004 AToL PI workshop, there has been growing interest in coordinating informatics research for botanical AToLs. We propose here to integrate additional related informatics infrastructure through a collaborative web services framework that can be accessed through any number of online workbench applications. Workbenches can be viewed as user-interfaces functioning as clients to the web services framework. A web services framework provides the best opportunity to integrate new AToL resources with existing tools that are being developed in various AToLs, as well as BioCorder, CIPRes, MorphBank, UBio, and other bioinformatics tool development projects that are using or will be using web services. David Paterson and William Piel are submitting an AToL proposal to address interoperability and cross analysis capability among CIPRes, TreeBASE, UBio, and will also collaborate on integrating the Botany Browser (see letter). For the proposed liverwort AToL, we will focus resources on implementing the new component tools discussed below as web services with workbench interfaces, including the Botany Browser as a master workbench application demonstrating interoperability across multiple resources. Informatics resources developed under web services architectures provide distributed design flexibility, extensibility, and scalability. Each service would provide a specific data management (e.g., storage, updates, queries) or processing function (e.g., voucher management, image conversion and storage, sequence alignment), and can be linked to other services where dependencies exist. Web services protocols explicitly define parameters for data exchange and request processing, but are intended to interact with machines and not humans. The function of the workbench is to provide user interaction, generate machine readable requests for the web services, provide session management and control functions, and process the results into human interpretable format (reporting and visualization). Since each web service need not reside on a single server, or even on the same operating system, queries to services can be aggregated across multiple servers through one or more workbench layers. Web services exist in an open architecture, so anyone else could potentially build a custom workbench implementing our services.



Workbench Applications – Our own workbench applications will address the following objectives (1) extension of the Botany Browser to liverworts for archived voucher information and integration of accession data with morphological and molecular data and visualizing phylogenies, (2) managing nucleotide sequence and molecular data and data input among participants, and (3) creation of the "Geographic PD Explorer".

Botany Browser Extension – All green plants projects will generate a rich data set in a variety of forms, including sequences, morphological descriptions, observations and measurements, trees, data matrices, and images. In the Angiosperm AToL (NSF- 0431266) we are providing an informatics infrastructure that will bring these varied resources together on the user's desktop through an online application we call the Botany Browser. Functionality of this concept for botanical data was demonstrated by Co-PI Nico Cellinese (2002) in a prototype tool, FileTaxon. Data queries are federated across multiple data resources, but the complexity is transparent to the user. The system generates automated URL-based web links to various sites including the International Plant Name Index (IPNI), Tropicos, Lifemapper, BioGeomancer, GenBank, and TreeBase. The inclusion of liverworts represents a natural extension of this on-going project. In this proposal we plan to expand the Botany Browser to include resources specific to liverworts, some of which are currently being built (e.g., index of accepted names and synonymy) and to migrate this application into a robust and extensible web services framework. We will extend the morphological component of the Browser beyond the morphology interface tailored to angiosperms and develop a controlled vocabulary for liverwort morphology. This will be accomplished in part through collaborative development with MorphBank developers, by establishing a mirror site for MorphBank at Yale University, and implementation of Taxonomic Databases Working Group (TDWG) SDD standard that CIPRes has also adopted. As part of the angiosperm AToL, we are also currently reviewing phylogenetic visualization tools, including hyperbolic trees (covered by a U.S. patent held by Inxight Software), and open source alternatives include Zoomable User Interface (ZUI) tools (e.g., TaxonTree 2003; SpaceTree, 2003) and the Java library behind them (Piccolo, 2004), as well as TreeJuxtapose (2005). Here we are proposing to build the necessary infrastructure to implement a visualization workbench component to liverworts.

Nucleotide Sequence Workbench – This work will be fully collaborative with the Fungal ToL project (AFTOL) hosted at Duke (see letter from F. Lutzoni & R. Vilgalys). Data is stored in a common format to facilitate integration with the other institutions, and provides a standards-compliant storage facility for final integration into the larger Tree of Life initiative. AFTOL has plans to integrate components of their existing infrastructure as web services. We will facilitate this migration and, where possible we will expand resources to the Liverwort AToL and the broader ToL community. All sequences generated for the project will be stored and referable to the trace electropherograms (trace files will be archived by individual institutions). In addition, an automated contig-assembly system has been developed using the software Phred and Phrap (http://www.phrap.org/) to streamline data integration directly from the sequencing facility to the database. This interface will provide automated retrieval facilities to enable downloading of data in FASTA, GDE Flat, and GENBANK formats, and automatically generated alignments (via ClustalX) of multiple taxon/gene compliments will be available in NEXUS format. Storage of a current 'stable' alignment of each data partition will also be available. The ability to generate specific taxon/gene matrices will enable rapid monitoring of the current phylogenetic hypotheses and provide a quality check on the available data.

The PD Explorer – Various approaches have been taken to the estimation of biodiversity. Perhaps the simplest are measures of taxic diversity, estimated as the numbers of species, genera, or higher-level taxa. However, taxic diversity assumes that all units are equal in value and this clearly is not the case, as noted many times (Faith 1992, Harper & Hawksworth 1994, Humphries et al. 1995, Moritz & Faith 1998, Nixon & Wheeler 1992, Vane-Wright et al. 1991). Various approaches have been proposed to incorporate phylogenetic considerations into biodiversity estimates. Alternative measures of phylogenetic diversity (PD) emphasize topology, numbers of nodes, and/or branch lengths (e.g., Vane-Wright et al. 1991, Crozier 1992; Nixon & Wheeler 1992, Faith 1992, 1994, Walker & Faith 1995, Williams et al. 1994a,b; Posadas et al. 2001). Conceptual issues that surround the choice of metric for PD estimates

include the relative importance of anagenic vs cladogenic origins for new phenotypic traits, the value of unique trait combinations vs. maximizing single-trait diversity, and how to deal with evolutionary rate heterogeneity (Humphries et al. 1995). Estimates that take amount of evolution into consideration may be the best available predictor of so-called “feature diversity” (Faith 1992); i.e., variation in other features that may be functionally important. The extent to which gene trees incorporating branch lengths predict diversity in other traits depends on the accurate estimation of branch lengths and patterns of evolutionary rate heterogeneity among different clades and loci. The basic approach is to estimate that percentage of the total tree length that is attributable to different taxon partitions.

PD can be estimated for assemblages of taxa occupying a particular geographic area, community, ecological type (e.g., epiphytic liverworts), or a particular clade (e.g., the Jungermanniidae relative to all liverworts). PD patterns can be used to prioritize areas for preservation (Polasky et al. 2001; Rodrigues & Gaston 2002). The optimal selection of multiple reserves is a complex problem that may utilize so-called "greedy search algorithms" that take into account complementarity among sites (Faith et al. 2004). Some models incorporate both species richness and phylogenetic diversity (Önal 2003). For communities, PD estimates can incorporate abundances and dominance-diversity relationships as well as presence-absence data (Barker 2002). Comparisons between PD estimated from neutral (or near-neutral) markers and phenotypic "feature diversity" can reflect patterns of natural selection on phenotypic traits (Diniz-Filho 2004) and rates of phenotypic evolution (Owens & Bennett 2000).

We propose to develop a system for comparing patterns of species richness in liverworts with phenotypic and molecular components of phylogenetic diversity. Our PD Explorer will facilitate investigations of geographic, ecological, and taxonomic partitioning of biodiversity. Liverworts are an excellent group for development of this utility because it is clear that the lineage as a whole is extremely old, but it appears that there are recent radiations as well. We envision that our approach will be applicable to other groups of organisms, and we presently have the data with which to apply the same algorithms to mosses for comparison. In the peatmosses (*Sphagnum*), Northern South America is a conspicuous hotspot in terms of taxic diversity but contains only 35% and 23% of global peatmoss PD for a nuclear and plastid locus, respectively (Shaw et al. 2003). Randomly sampling trees from the Bayesian posterior probability distribution indicates that phylogenetic uncertainty is not a major source of error in PD estimates (Shaw et al. 2004).

The necessary ingredients for our PD Explorer are a best-estimate phylogenetic tree for all (or nearly all) liverwort genera, PD algorithms embedded in a web service, and a workbench interface. Application of the explorer might be as follows. A user wishes to ask if there is more [liverwort] biodiversity in New Caledonia, or Madagascar. One approach is to count the number of species or genera in each region. A complementary approach is to upload a list of genera, and request an estimate of PD (expressed as a percentage of total [liverwort] PD for each region). Additional tractable questions might include how much additional PD is provided by New Caledonia, beyond that contained in Madagascar; i.e., to what extent is the PD redundant between these regions? The PD Explorer workbench will integrate distribution data from vouchered material mined from our own web services and services provided by GBIF and others. We will also include data from taxonomic checklists, as available for regions around the world, so biodiversity levels and redundancy can be compared for taxonomic and phylogenetic estimates of biodiversity. Incongruence between inferences obtained from the estimates can itself be enlightening. "Museums" of biodiversity where extinction rates have been low might contain deep clades yielding high PD and species richness, whereas regions that are hotspots of speciation might have many species but low PD. Since our phylogenetic tree will be based on data from all three genomes, the PD Explorer will also permit comparisons between geographic patterns in nuclear, plastid, and mitochondrial diversity. In addition, our 38 character morphological data set will provide the unique opportunity to test hypotheses about the relationship between molecular and phenotypic diversity and diversification. Estimates of liverwort PD will be made at the generic level because sampling at the species level will of course be incomplete. Assuming that most genera of liverworts are monophyletic, generic-level estimates of PD should provide a good approximation of global PD patterns. Shaw, Goffinet and Cox presently have a generic level phylogeny for mosses and so the PD Explorer developed for this project can provide comparisons between geographic patterns in moss and liverwort PD. As phylogenetic analyses of other organismal groups are completed, our PD Explorer can be expanded so that additional comparisons will be possible.

G. Education and Outreach

Our project will contribute to scientific infrastructure by training postdocs, graduate students, and undergraduates in liverwort systematics and phylogeny, and more broadly in plant morphology, molecular evolution, and bioinformatics. In addition to annual meetings at which trainees will have opportunities to interact with each other and with all of the project PIs, we will encourage graduate and post-doctoral trainees to visit other PI labs to gain experience with the broad range of techniques and approaches included in this project.

This project will also deliver materials and information that will promote continuing research and education aimed at Assembling the Tree of Life. We propose to develop a WEB-accessible virtual herbarium and interactive key to include all liverwort genera. A substantive component of our outreach effort will target undergraduates by integrating our project with an NSF-funded REU-Site program at Duke in "Bioinformatics and Biodiversity," and by organizing a symposium on Resolving the Green Plant Tree of Life, couched at a level that will be accessible to an undergraduate audience. We will also target secondary school teachers through a workshop on the methods and conceptual paradigms of phylogenetic biology and the Tree of Life, and through internet-accessible teaching modules. The workshop will also be offered at the Botanical Society of America (BSA), Education and Outreach Forum. We will co-sponsor professional development events for undergraduates at the BSA meetings with the NSF Undergraduate Mentoring in Environmental Biology "Increasing diversity at annual Botanical Society of America meetings" (PI: Renzaglia). K. Renzaglia is the Director of the SIUC McNair Scholars Programs and NSF Louis Stokes Alliance for Minority Participation (including the Bridge to Doctorate Program) at SIUC and thus she networks with these federally funded programs nationwide. We will aggressively recruit individuals from underrepresented populations to the Tree of Life Initiative through the contacts, listserves, and events sponsored by these programs. In addition, Duke University's biomedical departments have offered the Summer Opportunities Research Program (SROP) for under-represented minority students, which has increased interest in summer research on Duke's campus and strengthened linkages with such HBCUs as North Carolina Central University, North Carolina A&T University, and Winston-Salem University. We are cognizant of the issues and obstacles faced by ethnic minorities and women, and we will strive to create a nurturing environment that maximizes persistence and achievement of students from underrepresented groups (Clewell et al. 1992; Seymour & Hewitt 1997; Swail et al., 2003). Through a strong mentoring network, students will be integrated into our laboratory teams and will be provided with interactions and experiences that will facilitate socialization and professional development.

Web Presentations –SEM, optical images, and associated information produced for this project will be included in a Web-accessible, voucher specimen database. Another integrated component of the database is information on scientific names at any taxonomic level, including nomenclature, synonymy, classification, and common names. We will also develop an online, interactive key to all liverwort families and genera. This is a collaborative project already in progress between the Field Museum and J. Pickering of the University of Georgia (see letter from Pickering and http://www.discoverlife.org/nh/tx/Plantae/Bryophyta/). SIU hosts a website devoted to liverworts, developed as part of a PEET project. Duke will host the website associated with this project. It will include information about the goals, methods, and progress, and will provide laboratory protocols for nucleotide sequencing and other molecular methods, including primer sequences for all loci in the targeted sequencing and long-range plastid genome analyses. One component of the project website will be an overview of liverwort phylogenetics in the context of the NSF AToL program at a level useful to high school students. All of our web resources will be linked under the Botany Browser umbrella.

Public education about NSF-Assembling the Tree of Life The Duke herbarium organizes an annual event attended by professional and amateur bryologists (The Blomquist Foray). We spend the weekend somewhere in the southeast talking science and examining plants in the field. Each year we ask someone to give a presentation of interest to the whole group. During the first or second year of this project, we will present an overview of our project (with lots of pretty liverwort pictures) to the group. This is an extremely effective outreach activity to reach parents, teachers, voters, school board members, and professionals who indirectly support the Tree of Life Program. Similar presentations will be made at the North Carolina Botanical Garden and at annual naturalist rallies in the southern Appalachians, including the Wildflower Pilgrimage at the Great Smoky Mountain Park.

Comprehensive materials for teacher education – We will organize a one-day workshop for secondary school teachers on Assembling the Tree of Life. The symposium will be organized through the Duke University Program in Education (see http://www.duke.edu/web/education/) and will include lectures on the conceptual basis of phylogeny reconstruction, the importance of understanding phylogenetic relationships, phylogenetic reconstructions for selected groups of organisms, AND a local field trip to observe phylogenetic diversity in nature. Our goal will be to provide teachers with the tools necessary to teach students how and why reconstructions are valuable (and that they are based on objective data and rigorous analyses), an appreciation for new insights from the AToL research agenda, and an excitement for understanding and preserving the biodiversity around us. In addition, we will develop a series of hands-on, inquiry-based modules, made available through the project website, to enrich the educational toolboxes available to elementary and secondary school teachers. Our modules will include simplified, illustrated guides to liverwort identification, protocols for growing liverworts, and posters illustrating life cycles, ecology, and structural diversity.

H. Broader Impacts

Research: understanding the early evolution of embryophyte gametophyte and sporophyte morphologies – Liverworts are a key group for understanding the earliest evolution of the green plant life cycle. They are the ideal group in which to address fundamental evolutionary questions such as: How are apical growth systems established from generalized, spheroidal body plans? Are antheridia and archegonia developmentally related? How have patterns of sporopollenin deposition and spore wall organization changed through evolutionary time? What modifications in placental organization are related to increasing independence of the sporophyte generation? Our research will also set the stage for improved understanding of molecular and biochemical features of early land plants. There is a high degree of molecular rate heterogeneity among lineages, as well as between organellar and nuclear genomes (Lewis et al. 1997; Forrest & Crandall-Stotler 2005). Preliminary investigations have shown that RNA editing in mitochondrial genes is present in some liverworts (e.g., the simple thalloid *Pellia)*, but absent in others (e.g., the complex thalloid *Marchantia)* (Malek et al. 1996; Steinhauser et al. 1999). Lewis et al. (1997) suggested that there are no RNA editing sites in the plastid *rbc*L gene of liverworts, in contrast to mosses and hornworts.

A comprehensive phylogeny would allow workers to identify appropriate model liverworts for evolutionary studies of physiological and biochemical pathways, such as auxin metabolism (Cooke et al. 2002; Poli et al. 2003), desiccation tolerance (Oliver & Bewley 1997) and kinetic properties of enzymes like RubisCO (Raven 2000). It has been documented that liverworts produce a diverse array of secondary metabolites that display various types of biological activity, including antimicrobial, antiherbivory, and antitumor activity (e.g., Asakawa 1998, 1999; Frahm & Kirchhoff 2002). Advances in chemical separation techniques that require only small amounts of plant material have led to renewed interest in tapping the reservoir of potential pharmaceuticals found in liverworts (Banerjee 2001). Once a robust phylogeny has been resolved, the vast store of secondary compound data that exists can be mapped onto the phylogeny, thereby greatly facilitating pharmaceutical bioprospecting.

Training – The proposed project will involve some 5-7 postdoctoral associates and 7-9 graduate students. The impact on graduate training is substantial. In addition, we will collaborate with scientists from several other countries and invite students and/or postdocs to work with us directly on aspects of the research. These interactions benefit both the foreign trainees and U.S. participants by adding cultural and scientific diversity to the immediate project, and promote future collaborative research. Finally, we have a tangible commitment to promoting undergraduate participation in Assembling the Tree of Life. One of the major themes of our project is synergistic integration with other projects.

Outreach – Multiple and substantive outreach activities are associated with this project, including web-accessible interactive keys to all liverwort genera with photographic documentation of ultrastructural, anatomical, and morphological characters across the liverwort tree of life. The project website will provide information about our laboratory and analytical protocols and the Botany Browser will integrate all the components of our phylogenetic, molecular, and morphological research through our workbench applications. The new PD Explorer will provide a useful tool for all biologists interested in biodiversity patterns. We especially target post-doctoral scientists, graduate students, undergraduates, and high school (or younger) students, and direct additional substantive efforts toward secondary school teachers

and the public. Our continued development of live culture collections will provide a resource for the scientific community.

I. Results of Prior NSF Support

Jonathan Shaw: DEB-0089131, “Collaborative: Phylogenic and geographic patterns in moss diversity”, 2001-2004, $335,444; DEB-00-75611, 2000-2003, $225,000, “Phylogeny and biogeography of peatmosses (*Sphagnum*)”. The first award supported phylogenetic analyses of mosses based on multilocus DNA sequences. 16 papers have been published plus three currently in-press. A postdoc, two graduate students, and one undergraduate were supported by the award. Four European collaborators visited to Duke. The second award supported molecular analyses of *Sphagnum* at the generic, species, and population levels. Six papers been published to-date and one is in-press. One graduate student, an undergraduate, and two European collaborators were supported.

Bernard Goffinet: (DEB0089633) "Collaborative: Phylogenetic and Geographic Patterns in Moss Diversity", 2001-2004. (Collaborative with Duke DEB-0089131) See above; Human Resources at UCONN: 3 graduate RAs (2 women); 2 European visitors.

Barbara Crandall-Stotler and Raymond E. Stotler: DEB-9521883, "Monographic and Phylogenetic Investigations of the Fossombroniineae (Hepaticophyta)," 1995-2001, $612,675; DEB-9977961, Monographic and Phylogenetic Studies of Simple Thalloid Hepatics (Jungermanniopsida, Metzgeriidae), 2000-2005, $773,600." The first award supported monographic studies of one of the most speciose suborders of simple thalloid liverworts; to-date, 11 papers have been published, plus 2 currently in press and 1 in review. 7 M.S. theses, 3 Ph.D dissertations and 8 undergraduate research projects were supported by this award. The second award supported monographic studies of 16 genera of simple thalloid liverworts, and a multilocus phylogenetic analysis of the Metzgeriidae. To-date 4 papers have been published, 2 are in press, and 1 is in review; the research of 5 M.S. and 1 PhD students, 2 post-doctoral fellows, 9 undergraduates and 1 Latin American collaborator have been supported by this grant.

Yin-Long Qiu: DEB 0093012 (Early CAREER award), “Mitochondrial genome evolution and land plant phylogeny”, 2001-2005, $600,000; DEB 0431239, “AToL: The angiosperm tree of life: resolving the trunk of the tree and 12 of its thorniest nodes”, 2004-2009, $244,948. The first award supported a multigene and genomic structural analysis of nonflowering land plants, and an investigation of mitochondrial genome evolution in early land plants. Four papers have been published and one has been submitted. Five postdocs, one graduate student, and several undergraduate students have been partially supported by the award. The second award will start in the summer of this year.

R. Beaman & N. Cellinese (with M. Donoghue, B. Heidorn, B. Thiers, H. Rolen, and M. Tulig). “Collaborative Research: Rapid Digital Specimen Image and Data Capture: A Web Services Solution.” DBI-0345341; $845,775 (total to three institutions) 04/01/2004 – 03/31/2009. This project offers proof of concept and an initial implementation of ‘one-button’ specimen imaging and data capture. We are currently testing the image conversion and Optical Character Recognition web services. R. Beaman & N. Cellinese (with D. Soltis, P. Soltis, W. Judd, S. Manchester, M. Donoghue, L. Hickey, R. Olmstead, Y.L. Qiu, K. Sytsma, C. Davis, K. Hilu, and M. Sanderson). “AToL: Collaborative Research: Resolving the Trunk of the Angiosperm Tree and Twelve of its Thorniest Branches.” NSF-0431242; $3,003,000 (total to eight institutions) 09/01/2004- 08/31/2009. We are in the early stages of developing the Botany Browser, a comprehensive navigation system that will integrate multiple resources.

Karen Renzaglia: DEB- 0228679, “Collaborative Research: Deep green plant phylogenetics: novel analytical methods for scaling data from genomics to morphology.” (with O’Kelly, Mandoli, Wolf, Mishler, Boore, Olmstead and Donoghue), 2002-07, $2,751,811. and DEB-0235985 “Collaborative Research: Biodiversity, phylogeny and biogeography of hornworts”, (with Duff), 2003-2006, $210,703. The first is a combined molecular and morphological approach to resolving the primary pattern of evolutionary diversification among green plants. Seventeen papers and six chapters in books have been published thus far. Seventeen scientists from around the world have collaborated on the project. The second award supports morphological and molecular phylogenetic analyses of hornworts. Five papers have been published to-date and three have been submitted. One Australian and two European researchers have worked on the project. The Renzaglia lab has supported one technician, three graduate student, six undergraduates and one high school teacher on these combined awards.

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