The Efficacy of the DNA Barcoding Proctol in determining species in the red algal orders Batrachospermales and Thoreales and a comparison with the plastid *rbc*L gene.

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

The red algae (Rhodophyta) is a monophyletic phylum and is comprised of seven classes including the Florideophyceae. The class monophyletic Florideophyceae is postulated to have diverged from the class Bangiophyceae and contains over 32 orders including the freshwater Batrachospermales and Thoreales. Classifications within these orders as well as the class are based predominantly on female reproductive characters and vegetative morphology. The order Batrachospermales contains a number of families and genera with the genus *Batrachospermum* being the largest with eight recognized sections. The order Thoreales was once considered a genus within the order Batrachospermales but is currently recognized as an autonomous order. Due to the cryptic nature of the genera, particularly *Batrachospermum,* the Morphological Species Concept has proven to be limiting in the classification at the species level. This study examines the usefulness of the mitochondrial gene encoding cytochrome c oxidase subunit I (COI) in delimiting species within the orders Batrachospermales and Thoreales from several countries spanning three continents and comparing this data to a parallel analysis of the gene encoding the large subunit of the chloroplast enzyme Ribulose 1, 5-bisphosphate carboxylase/oxygenase (*rbc*L). Sequence data and phylogenetic analysis illustrates possible delineation among species using the COI marker. Distinct clades of sections *Batrachospermum, Setacea, Virescentia, Turfosa, Contorta, Gonimopropagulum* and *Aristata* were observed from various geographic locations; as well as clades of genus *Sirodotia, Tuomeya Lemanea, Paralemanea* and *Thorea*. The present study proposes the elevation of the current recognized infrageneric sections to the status of genus based on both COI and *rbc*L genes sequence data. In addition, very few clades appeared to reflect any biogeographic trends.

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1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

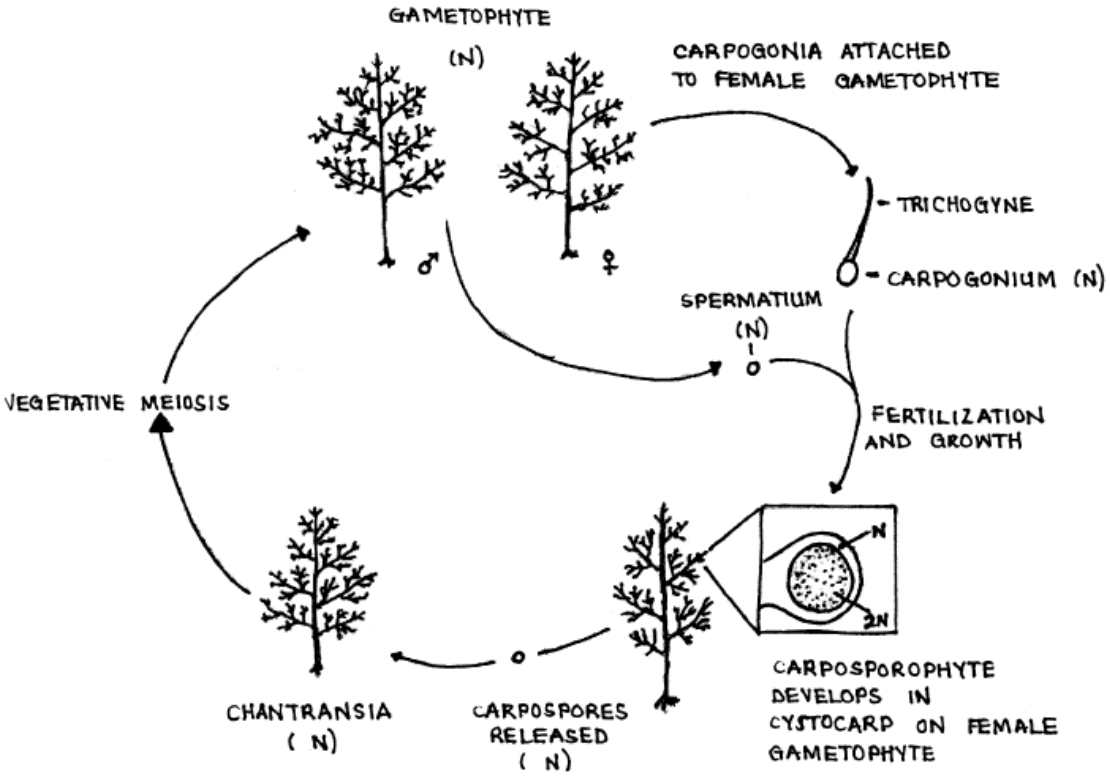
The red algae (Rhodophyta) are the oldest taxonomically defined eukaryote represented in the fossil record (Andersen, 1992; Knoll, 1992; Butterfield, 2000) and currently contains approximately 6000 – 10,000 extant species (Glimn-Lacy and Kaufman, 1984; Woelkering, 1990; Thomas, 2002;) in over 834 genera (Schneider and Wynne, 2007). In addition, the Rhodophyta are a distinct monophyletic lineage characterized by a two-membraned plastid that contains the photosynthetic pigments phycocyanin, phycoerythrin and allophycocyanin, which are organized in phycobilisomes in unstacked thylakoids (Gabrielson *et al.*, 1985; Gabrielson and Garbary, 1986; Gabrielson *et al.*, 1990). The Rhodophyta are also characterized by the lack of flagella, centrioles and the photosynthetic pigments; chlorophyll b and chlorophyll c (Woelkering, 1990; Freshwater *et al.*, 1994; Yoon *et al.*, 2006). Additional features observed in some, but not all, red algae include pit connections (pit-plugs) and secondary pit-connections that separate cells (Wetherbee and Quirk, 1982; Gabrielson *et al.*, 1985; Gabrielson *et al.*, 1990; Saunders and Hommersand, 2004); mitochondria associated with the development of golgi bodies; and plastids surrounded by thylakoids (Adam *et al.*, 2005). In addition, some red algae also exhibit a complex life history (Figure 1) that involves three phases: gametophyte, carposporophyte and chantransia (John *et al.*, 2002; Kumano, 2002; Rajan, 2002).

Traditional classification of the Rhodophyta considered this group to consist of one class (Rhodophyceae) and two subclasses (Florideophycidae and Bangiophycidae) (Gabrielson *et al.*, 1985; Gabrielson and Garbary, 1986; Gabrielson *et al.*, 1990; Freshwater *et al.*, 1994, Ragan *et al.*, 1994). However, more recent studies (e.g. Yoon *et al.*, 2006) have re-evaluated this and divided this phylum into seven classes including: the Rhodellophyceae, Cyanidiophyceae, Compsopogonophyceae; Stylonematophyceae,

Porphyridiophyceae; Bangiophyceae; and Florideophyceae (Saunders and Hommersand 2004; Yoon *et al.*, 2006). The class Florideophyceae is hypothesized to have arisen from the class Bangiophyceae (Oliveira and Bhattacharya, 1999; Müller *et al.*, 2001b) and the Florideophyceae is clearly a monophyletic group (Gabrielson *et al.*, 1985, Gabrielson and Garbary, 1986; Gabrielson *et al.*, 1990; Freshwater *et al.*, 1994, Ragan *et al.*, 1994). This class contains over 32 orders (Schneider and Wynne, 2007) including two that are the focus of this study: Batrachospermales Pueschel et Cole, and the Thoreales Müller, Sheath et Sherwood. Classification within the Florideophyceae is based predominantly on female reproductive characters and vegetative morphology (Freshwater *et al.*, 1994). Nonetheless, recent categorizations have employed comparisons of ultrastructure pit-connections or pit-plugs characters that are synapomorphic (Kapraun *et al.*, 2007) as well as molecular phylogenetic analyses of various genes (e.g. Saunders and Kraft, 1997; Harper and Saunders,2001; Le Gall and Saunders, 2007) . With respect to pit-plug characteristics, orders within the Florideophyceae have been categorized into two groups based on the number of cap layers (0, 1, or 2) and the presence or absence of cap membranes (Pueschel and Cole 1982; Pueschel, 1989). Orders with two cap layers include the Acrochetiales, Balliales, Balbianiales, Batrachospermales, Corallinales, Colaconematales, Nemaliales, Palmariales, and Thoreales (Saunders and Bailey, 1997; Harper and Saunders, 2001) and single cap layer, Hildenbrandiales (Freshwater, 1994; Harper and Saunders, 2001). Those exhibiting no cap layers but rather cap membranes, include the Gigartinales, Ceramiales, Bonnemaisoniles, Gracilariales, Halymeniales, Nemastomatales, Rhodymeniales, and Plocamiales (Freshwater *et al.*, 1994; Saunders and Bailey, 1997; Saunders and Hommersand, 2004).

Figure 1: Life Cycle of *Batrachospermum*

The life cycle shown above illustrates the triphasic, heteromorphic life cycle of *Batrachospermum*. The three life phases; gametophyte, cystocarp and chantransia all of which are haploid, characterize this life history. In this life cycle, the gametophytic plant develops male (spermatangia) and/or female (carpogonia) sex organs which bear spermatium ♂and carpogonium ♀. By attaching to the trichogyne, the spermatium fertilizes the carpogonium. As a result of fertilization, a carposporophyte is developed from the carpogonium. Following this, the carposporophyte produces carpospores which upon release germinate into a heterotrichous chantransia stage. Filaments of the chantransia by vegetative meiosis produce new haploid gametophytes. Reproductive characteristics of this history are often used in the classification of members of the Rhodophyta.

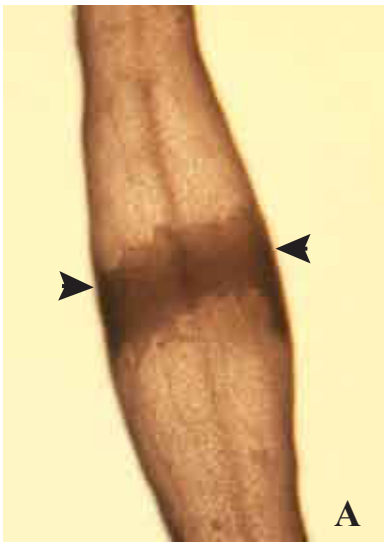

GAMETOPHYTE
(N)
CARPOGONIA ATTACHED
TO FEMALE GAMETOPHYTE
TRICHOGYNE
CARPOGONIUM (N)
SPERMATIUM
(N)
FERTILIZATION
AND GROWTH
VEGETATIVE MEIOSIS
N
2N
CARPOSPOROPHYTE
DEVELOPS IN
CYSTOCARP ON FEMALE
GAMETOPHYTE
CHANTRANSIA
(N)
CARPOSPORES
RELEASED
(N)

1.2 THE ORDER BATRACHOSPERMALES PUESCHEL ET COLE

The order Batrachospermales Pueschel et Cole (1982) was once classified as a family within the order Nemaliales (Gabrielson *et al.*, 1990; Pueschel, 1994) and is now recognized within it's own order, the Batrachospermales. This order contains three families: Batrachospermaceae (four genera: *Batrachospermum, Nothocladus, Sirodotia* and *Tuomeya*), Psilosiphonaceae (one genus: *Psilosiphon*) and Lemaneaceae (two genera: *Lemanea* and *Paralemanea*) (Kumano, 2002; Wehr and Sheath, 2003). The genus *Batrachospermum* is the largest of the four genera within the Batrachospermaceae, comprising eight recognized sections: *Contorta, Setacea, Virscentia, Arista, Turfosum, Hybrida, Batrachospermum* (Necchi, 1990; Kumano, 2002; Wehr and Sheath, 2003) and *Gonimopropagulum* (Sheath and Whittick, 1995; Kumano, 2002). Necchi and Entwisle (1990) suggested that the three other genera, *Nothocladus, Sirodotia* and *Tuomeya*, be placed in the genus *Batrachospermum* as sections as the morphological characters employed to differentiate them were not considered sufficient for generic standing. Nonetheless, Vis *et al.* (1998) proposed to maintain their generic status since some morphological characters noted in these genera are not observed in the genus *Batrachospermum*. The order Batrachospermales is exclusively freshwater with its members described as paraphyletic, lacking tetrasporangia and auxiliary cells, with two cap-layered pit plugs (Figure 3 a – f) and spore development that is similar to that of genus *Nemalion* (Sheath, 1984; Gabrielson and Garbary, 1986; Vis *et al.*, 1998).

Figure 2: Images of Taxa Studied Showing Reproductive Structures.

A) *Paralemanea* sp. thallus showing distinct ring of spermatangia (arrowheads), B) *Lemanea* sp. portion of a gametophyte thallus with spermatangia patches (arrowheads), C) *Batrachospermum* sp., whorl with carposporophytes (triple arrow head) within it, D) *Paralemanea* sp., cross-section showing outer cortex layer and carposporophyte (triplearrows), E) *B. involutum*, carpogonium showing club-shaped trichogyne, F) *Batrachospermum* sp., fascicle showing filaments, G) *Thorea* sp. showing thallus with dense central medulla, loosely arranged assimilatory filaments (arrowheads) , H) *Thorea hispida*, showing thallus with dense central medulla, loosely arranged assimilatory filaments (arrows) and numerous secondary branches (arrowheads).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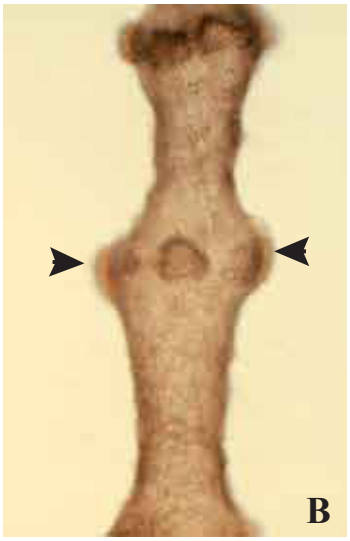
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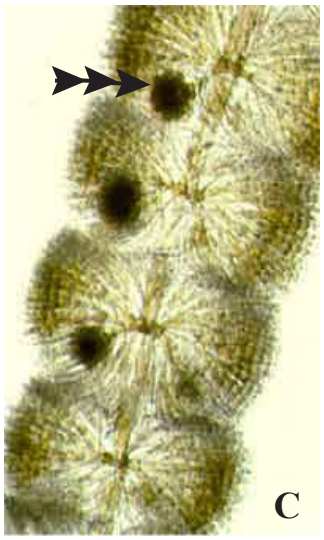
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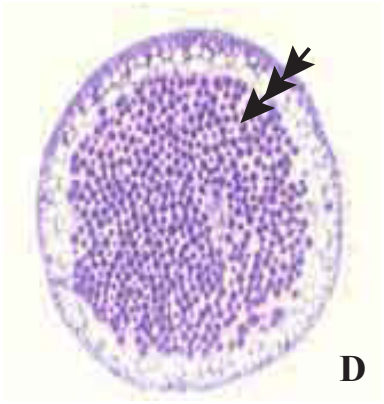
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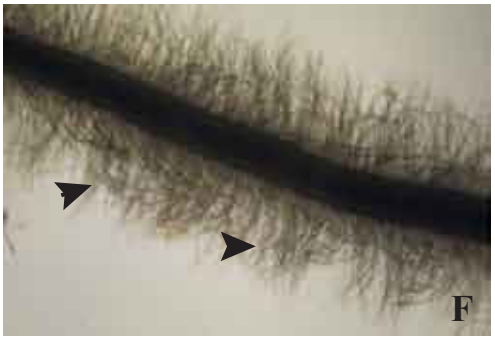
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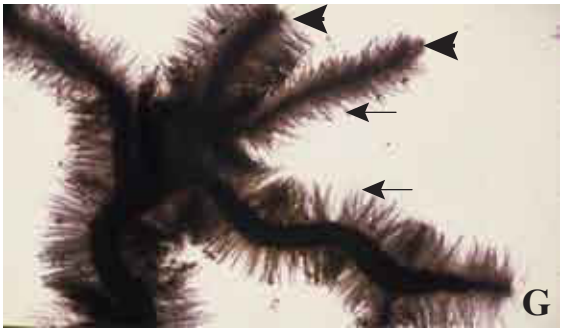
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H

1.3 FAMILY BATRACHOSPERMACEAE FRIES

The Family Batrachospermaceae is described as a cosmopolitan family with four genera *Batrachosperum, Sirodotia, Nothocladus* and *Tuomeya* (restricted to the Northern hemisphere) and over 100 species (Entwisle *et al.*, 2007). This family is characterized by gametophyte with cylindrical to moniliform outline, as well as uniaxial with regular whorls for laterals that are branch determinate. Axial filaments are observed to be broad with periaxial cells usually between 4 to 6 cells (Kumano, 2002; Entwisle *et al.*, 2007)

1.4 GENUS *BATRACHOSPERMUM* ROTH

Initially described by Roth in 1797, the genus *Batrachospermum* occurs worldwide, from the tropics to the Arctic, in streams often attached to rocks or other macrophytes (Sheath and Cole, 1993; Vis *et al.*, 1994, Branco and Necchi, 1997; Necchi and Branco, 1999). The genus *Batrachospermum*, with respect to the rest of the genera in the order Batrachospermales is regarded as paraphyletic (Vis *et al.*, 1998; Kapraun *et al.*, 2007) and consists of several distantly related taxa that are morphologically similar (Vis *et al.*, 1998; Vis and Entwisle, 2000). The genus *Batrachospermum* is distinguished by the following characters: carpogonia branches that arise from fascicle and periaxial cells, carpogonia that are somewhat asymmetric or symmetric with club-shaped or elongated trichogynes, and determinate carposporophyte with gonimoblast filaments that are radially-branched (Sheath 1984; Kumano, 2002). The life history of *Batrachospermum* (Figure 1) is dominated by the gametophyte, with its morphological characters like size of whorl, carposporophyte diameter, proximal cell length and width among others, being used repeatedly in species discrimination (Entwisle *et al.*, 2004). This genus demonstrate an oogamy form of

reproduction, in which a non-motile male reproductive cell (spermatium) fertilizes a female reproductive cell (carpogonium) that is significantly larger than the male reproductive cell itself (Kumano, 2002). In contrast to most members of the class Florideophyceae, the genus *Batrachospermum* lacks a tetrasporic meiosis stage in its life history; nonetheless, it has one gametophyte stage and two sporophyte stages (Figure 1)(Sheath, 1984; Kumano, 2002). The genus *Batrachospermum* is currently divided into approximately eight infrageneric sections; *Batrachospermum, Contorta, Setacea, Virescentia, Aristata, Turfosa, Hybrida*, and *Gonimopropagulum* (Sheath and Whittick, 1995; Kumano, 2002; Müller *et al.*, unpublished). The division of members of the genus *Batrachospermum* into sections was first demonstrated by Sirodot (1873) to constitute four sections based on differences in morphology of the carpogonium and trichogyne. Nonetheless, the current eight sections are distinguished from one another primarily on other features such as morphology of carpogonial branch (size and shape), relative whorl size, carposporophyte structure, trichogyne shape, and presence of unique propagules (Reis, 1974; Kumano, 1993, 2002; Sheath and Whittick, 1995; Stewart and Vis, 2007; Entwisle and Foard, 2007). The eight sections are described as follows:

Section *Aristata* Skuja: well-developed whorls, carpogonial branches that are straight, extended, and separated from fascicles, whorls with pedicellate and spherical carposporophytes (Sheath et al, 1994a; Kumano, 2002). Nevertheless, to differentiate species within this section characters such as primary fascicle cell number, diameter of whorl, carposporangium size, trichogyne shape, secondary fascicle density, and length and localization of involucral filaments are used (Kumano, 2002).

Section *Batrachospermum* DeCandolle: straight undifferentiated carpogonial branches (developed from both fascicle and pericentral cells); well-developed whorls; carpogonia with trichogynes (club- to urn- shaped) and small; globose, pedicellate carposporophytes (observed at different distances from the axis of the whorl) (Kumano 1993; 2002; Vis *et al.*, 1995).

Section *Contorta* Skuja: characterized by carpogonial branches twisted in a helix form, well-developed confluent whorls, sessile or stalked trichogynes (Flint, 1949; Sheath *et al.*, 1992; Shulian and Zhixin, 2005). Kumano (1993) proposed dividing section *Contorta* into 5 subsections*: Intorta, Torrida, Procarpa, Kushiroense, Ambigua*. Sheath *et al.* (1992) divided this section into 5 distinct groupings based on both qualitative and quantitative characteristics.

Section *Gonimopropagulum* Sheath and Whittick: characterized by well-developed whorls, oboconical elongated trichogynes, differentiated carpogonial branches that are straight, sessile carpogonia, absence of carposporophyte, presence of unique propagules partitioned into regions by the septum (Sheath and Whittick, 1995; Kumano, 2002).

Section *Hybrida* De Toni: well-developed whorls, axial and globular carposporophytes, elliptical or ovoid trichogynes and carpogonia with sessile (Necchi, 1990; Sheath and Vis, 1995; Kumano, 2002).

Section *Setacea* De Toni: characterized by short whorls made up of few fascicles closely pressed to the main axis, short carpogonial branches arising from the pericentral cell, and globular carposporophytes extending beyond the reduced whorls. The section *Setacea* is differentiated from the section *Contorta* as having carpogonial branches that are straight instead of ones in helix form (Sheath *et al.*, 1993c).

Section *Turfosa* Sirodot: characterized by plants pseudo-dichotomously branched, straight carpogonium-bearing branches dropping from periaxial cells, reduced well developed wells, big spherical or semi-spherical carposporophytes with two types of gonimoblast filaments (radially branched determinate and prostrate inderterminate) (Kumano, 2002).

Section *Virescentia* Sirodot: well-developed whorls; straight and short carpogonial branches (arise from proximal fascicle cells or pericentral cells); carposporophytes produced singly or in pairs along the main axis; elongated carpogonia with cylindrical and pedicellate trichogynes (Mori, 1975; Starmach, 1977; Sheath *et al.*, 1994b; Kumano, 2002).

Table 1: Summary of Sections of Batrachospermum and Taxa

Section	Taxon
Aristata	*B. macrosporum*
Batrachospermum	*B. anatinum*
	B. arcuatum
	B. boryanum
	B. confusum
	B. gelatinosum
	B. heterocorticum
	B. involutum
	B. spermatoinvolucrum
	B. sporulans
Contorta	*B. ambiguum*
	B. globosporum
	B. intortum
	B. louisanae
	B. procarpum
	B. spermatiophorum
Gonimopropagulum	*B. breutelii*
Hybrida	*B. virgato-decaisneanum*
Setacea	*B. androinvolucrum*
	B. atrum
Turfosa	*B. turfosum*
Virescentia	*B. helminthosum*
	B. elegans

1.5 OTHER GENERA: *NOTHOCLADUS* SKUJA, *SIRODOTIA* KYLIN, *TUOMEYA* HARVEY

The genus *Tuomeya* was created by Harvey in 1858 and differentiated from the other genera of the order Batrachospermales by having a thallus that is densely branched, cartilaginous, and pseudoparenchymatous, with lateral whorls compacted and radiating from a uniseriate axis (Gordon, 1934; Kaczmarczyk *et al.*, 1992). Additional distinguishing features were included by Skuja (1944) and Webster (1958), and these include unique secondary gonimoblast with cylindrical cells (Kaczmarczyk *et al.*, 1992). Necchi and Entwisle (1990) treated the genus *Tuomeya* as a section of the genus *Batrachospermum* on the basis of taxonomic changes such as often ill-defined compactness of cortex, prominence of dome-like apical cell similar to ones observed in the genus *Nothocladus*, and asymmetrical distinct carpogonia that is of little use as a sectional differentiating character (Kaczmarczyk *et al.*, 1992). On the contrary, Kumano (1993) treated the genus *Tuomeya* and the genus *Batrachospermum* as separate genera. Kaczmarczyk *et al.* (1992) observed the formation of spermatangia at the apex of determinate branches in species of the genus *Tuomeya* an examined feature characteristic of other members of the Batrachospermales (Sheath, 1984). As observed by Skuja (1944) and Webster (1958), gonimoblast filaments are made up of cylindrical cells; however, Kaczmarczyk *et al.* (1992) observed branch systems that are more dense than described by Stechell (1890) and Webster (1958), incorrectly distinguished as branches of spermatangia. The carposporophytes of the genus *Tuomeya* are similar to those observed for the section *Contorta* in the genus *Batrachospermum* (Necchi, 1990; Sheath *et al.*, 1992). Infrageneric classification of approximately 24 populations by Kaczmarczyk *et al.* (1992) of the genus *Tuomeya* on the basis of morphometric features produced two groups for this genus. The two groups were separated primarily on the basis of gonimoblast cell number and plant length, nonetheless, these difference observed in these features offers no base for the separation of these two groups (Kaczmarczyk *et al.*, 1992).

The genus *Sirodotia* is distinguished from other genera by its asymmetrical carpogonium in addition to its off-center trichogyne attachment, semiglobular protuberance on one side of the base, as well as a carposporophyte with branched gonimoblast filaments inching along the main axis (Kylin, 1912; Sheath, 1984). Necchi and Entwisle (1990) treated the genus *Sirodotia* as a section in the genus *Batrachospermum* on the basis of *Sirodotia* having a distinct asymmetrical carpogonia, a feature that is observed to vary considerably in shape, hence, not suitable for sectional classification. Nonetheless; Kumano (1993) retained the genus *Sirodotia*, separate from the genus *Batrachospermum* on the basis of difference in reproductive structures, with the genus *Sirodotia* possessing a diffuse gonimoblast and a lobed carpogonium base compared to the genus *Batrachospermum* with dense globular gonimoblast and an isodiametric carpogonium base (Kumano, 1993). Necchi *et al.* (1993) reported fourteen species of *Sirodotia*, identified on the foundation of shape and size of whorl, carpogonium, fascicle cells, and the occurrence of specialized spermatangial branches (Flint, 1948; Kumano, 1982c). Necchi *et al.* (1993) also proposed that asymmetric carpogonia with off-center trichogynes, as well as the occurrence of a distinct basal protuberance, and the formation of a diffuse, filamentous, carposporophyte that broaden longitudinally along the main axis are both unique characters in the family Batrachospermaceae. Consequently, the authors suggested that a detailed morphometric analysis of numerous members of the family would be essential to establish whether these two characters are simply an excessive in the range of morphological variation within the family (Necchi Jr. *et al.*, 1993). Necchi Jr. *et al.* (1993) proposed the genus *Sirodotia* be retained as a distinct taxonomic unity until the above is done.

The genus *Nothocladus* has members with typical batrachospermalean pit-plug with two cap layers, of which the outer one is dome-shaped (Sheath *et al.*, 1996a). The genus was created by Skuja (1934) to include populations of the order Batrachospermales that have well-developed whorls that are confluent and lateral, mucilaginous and cartilaginous filaments, corticated central axis among others characters (Sheath *et al.*, 1996a). The taxonomic significance of the three key features; symmetrical carpogonia,

diffuse carposporophyte (determinate) and specialized spermatangial filaments that distinguish the genus *Nothocladus* are uncertain (Sheath *et al.*, 1996a). For example Starmach (1977) concluded after observing species such as *Nothocladus* may be apogamic, that is with only female filaments occurring. As well, for the presence of indeterminate gonimoblast filament and symmetrical carpogonia, Necchi and Entwisle (1990) included *Nothocladus* to the section *Turfosa* of the genus *Batrachospermum*. Nonetheless, Kumano (1993) maintained *Nothocladus* at the generic level.

1.6 FAMILY LEMANEACEAE ROEMER

The family Lemaneaceae, like other families in the order Batrachospermales has a heteromorphic triphasic life cycle made up of the phases; chantransia, gametophyte and carposporophyte (Magne, 1967; Sheath, 1984). Traditionally, all freshwater red algae samples that possess uniaxial cartilagineous and pseudoparenchymatous thalli are placed under this family (Kučera and Marvan, 2004) with other characteristics such as fascicles cells with large vacuoles that occupy most of the cell volume towards the interior. The family Lemaneaceae is divided into two genera *Lemanea* and *Paralemanea* (Kučera and Marvan, 2004, Xie *et al.*, 2004). The genus *Lemanea* is characterized by plants with axial but no cortical filaments, as well as hair cells in the inner cortex and ray cells shaped like T- or L- and closely applied to the outer cortex. Arrangement of nodal spermatangial sori are observed as patches (Figure 2B). On the other hand, members of genus *Paralemanea* are characterized by the lack of stalks and hair cells in the inner cortex of the plants. Axial filaments of these plants are enclosed by cortical filaments, while their ray cells are made up of two layers, the proximal layer which does not touch the outer cortex and the distal layer which branches like a "Y" and connects to the cortex. Unlike the genus *Lemanea*, nodal spermatangial sori arrangement of the genus *Paralemanea* is in the form of rings (Figure 2A)(Xie *et al.*,

2004). Members of the family Lemaneaceae are observed in streams, largely distributed in northern hemisphere (Sirodot, 1872; Atkinson, 1890; Isrealson, 1942; Khan, 1973, Vis and Sheath, 1992) and some few observed in southern South America (Necchi and Zucchi, 1995). Taxonomic classification of this family has held different views from several authors. For example Sirodot (1872) divided the *Lemanea* into two genera according to both morphological and anatomical characters. Sirodot proposed including in the genus *Sacheria* species that are free of axial cortication and have T- or L- shaped rays cells near the outer cortex. On the other hand, Sirodot proposed the genus *Lemanea* to include species with axial cortication and simply ray cells that are not adjacent to the outer cortex. On the contrary, in 1959, Silva proposed that the genus (subgenus) *Lemanea* include species that lack axial cortication and the genus (subgenus) *Paralemanea* include species that have axial cortication.

1.7 FAMILY PSILOSIPHONACEAE ENTWISLE, SHEATH, MÜLLER AND VIS

Previously assigned to the family Lemaneaceae, is been shown to be distinct phylogenetically on a genetic level from the two genera *Lemanea* and *Paralemanea* in that family. The family Psilosiphonaceae is characterized by a cartilaginous thallus, uniaxial with determinate laterals like that of the family Batrachospermaceae, fascicle structure that is obscure and an outer cortex that is pseudoparenchymatous (Kumano, 2002; Entwisle, 2007).

1.8 THE ORDER THOREALES SHEATH, MÜLLER AND SHERWOOD

Once considered a genus within the order Batrachospermales, the new family Thoreaceae is a well-supported monophyletic clade within the new order Thoreales (Müller *et al.*, 2002; Harper and Saunders, 2001). The family Thoreaceae is observed worldwide; however, likely to be observed in temperate warm waters or tropical and subtropical regions (Sheath *et al.*, 1993a). Members of this order are exclusively freshwater, characterized by members with uniaxial chantransia stage, gametophytes with multiple axials, and two cap-layered pit plugs (Kapraun *et al.*, 2007). Studies by Müller *et al.* (2002) observed a monophyletic cluster of Thoreaceae from the Lamneaceae and the Batrachospermaceae; the other two families categorized within the Batrachospermales. An analysis of DNA sequences and ultrastructure examination brings up uncertainty about the classification of the Thoreaceae in the Batrachospermales (Müller *et al.*, 2002). Further, examination of Thoreaceae revealed 18S rRNA secondary structure components not observed in other members of the Rhodophyta (Müller *et al.*, 2002). As well, the pit-plugs (gametophytic and chantransia stages) of the Thoreaceae are made up of two cap layers, a common feature of the Batrachospermales, no pit-plug membranes were confirmed in the Thoreaceae, hence indicating further that the Thoreaceae have been placed in the wrong order, the Batrachospermales and must therefore have it own order, the Thoreales (Müller *et al.*, 2002).

The solely freshwater order Thoreales was created by Müller *et al.* (2002) to include the genus *Thorea* (Sheath *et al.*, 1993a; Müller *et al.*, 2002) with short branch monosporangia (Sheath *et al.*, 1993a) separating it from Nemalionopsis genus with monosporangia at the tips of assimilatory filaments confined at the periphery of the thallus (Sheath *et al.*, 1993a). The phylogenetic relationship and taxonomy of the Thoreaceae to additional lineages of the red algal is not entirely resolved to date. For example *Thorea hispida* as observed by Schnepf (1992) have plate-like shaped pit-plugs without cap layers compared to a domed-shaped pit plug outer cap layer observed for *Thorea violacea* by Lee (1971). The pit-plug outer

cap layer is a characteristic used to distinguish Batrachospermales (Pueschel, 1994), hence any discrepancies in shape renders this feature futile for classification purposes. Pit plugs with no cap membranes have not been observed in either the *Thorea* or the *Nemalionopsis* (Müller *et al.*, 2002). Further studies of the Thoreaceae using ultrastructure and analysis of sequences from different gene regions need to be conducted to resolve the taxonomic issues of the Thoreales.

1.9 THE MORPHOLOGICAL SPECIES CONCEPT (MSC): APPLICATION TO THE BATRACHOSPERMALES AND THOREALES

The MSC is probably the most commonly used methodology to delineate taxa within the red algae; however, as noted with many other groups, the use of morphology can be problematic but should not be ignored. Among members of the order Batrachospermales, the morphospecies concept has in some taxonomic studies proven to be quite useful when applied alone or combined with phylogenetic analyses. For example Vis and Sheath (1998) combined gene data (*rbc*L and ITS1, ITS2) with morphometric data in reducing *B. spermatoinvolucrum* to a form of *B. gelatinosum*, which encompasses the characteristic carpogonial involucral filaments bearing spermatangia. Similarly, Müller *et al.* (2008, submitted) combined carpogonial branch morphology with phylogenetic analyses of the chloroplast *rbc*L gene and the mitochondria cox2-3 spacer in strengthening the differentiation of infrageneric taxa under genus *Batrachospermum*. As well, well-defined morphological characters were employed by Sheath *et al.* (1992; 1993c; 1994a, b, c) Vis and Sheath (1996) and Vis *et al.* (1995; 1996a, b), in circumscribing each sections under the genus *Batrachospermum*. Despite its benefits, it is clear that the use of morphology alone in traditional taxonomic practices is not enough to provide accurate and reliable taxonomic information. Limitations of the MSC has been observed within and among red algal species with extreme morphological variability and often lacking the obvious features (synapomorphies) used in identifying

them (Hebert *et al.*, 2003a, Robba *et al.*, 2006). For example, observations made in Rhodophyta species such as *Bangia* and *Porphyra*, indicate that morphology alone is not sufficient for discriminating between these genera (e.g. Brodie *et al.*, 1998; Müller *et al.*, 1998; 2005). Consequently, morphology can be misleading when attempting to differentiate species with a long evolutionary history like ones belonging to the Rhodophyta, many of which have different generation alternating forms (Robba *et al.*, 2006). Categorizing taxa within the order Batrachospermales has been subjected to several changes, especially with members of the genus *Batrachospermum*. Currently, molecular data available for this genus conflicts with morphological features traditionally employed to classify the species within this genus (Kapraun *et al.*, 2007), therefore, a comprehensive re-examination of the species within the genus *Batrachospermum* is required.

At the infrageneric level, taxons within the confines of section *Batrachospermum* have been characterized on the grounds of whether thallus were monoecious or dioecious; shape of their whorls; their carpogonia shape and size; monosporangia; and the presence or absence of spermatangia (on the involucral filaments of the carpogonial branch) (Vis *et al.*, 1995). Nonetheless, it is hypothesized that some of these features are impacted by conditions of the environment (Israelson, 1942). In a study by Necchi (1990), Necchi observed the presence of both sessile and pedicellate trichogyne in a number of the *Batrachospermum* species he examined and proposed this character as not valid for sectional classification. Lately, several species have been characterized using measures such as inflated and irregular cortication, carpogonial branches with involucral fascicle developed on one side, and cylindrical-shaped fascicle cells (Vis *et al.*, 1995). Several authors including Necchi and Entwisle (1990) and Vis *et al.* (1998) have demonstrated uncertainty in the criteria's employed in distinguishing taxa at the generic and infrageneric level in the order Batrachospermales. For example, the section *Setacea* is differentiated from the section *Contorta* as having carpogonial branches that are straight instead of ones in helix form (Sheath *et al.*, 1993c; Vis *et al.*, 1998). Previously, the section *Setacea* was incorporated in

the section *Virescentia* by Necchi (1990) and Necchi and Entwisle (1990), however both authors continued separating taxa with well-developed whorls and reduced whorls (Sheath *et al.*, 1994a).

Similar to the section *Batrachospermum*, species within the section *Virscentia* have been differentiated using characters such as monoecious or dioecious, length of carpogonial branch and localization, density of secondary branches and terminal hairs, and the shade of green (Mori, 1975; Starmach, 1977; Kumano, 2002). The above species traits however are observed to be variable; hence, most species are defined inadequately (Sheath *et al.*, 1994b). As well, numerous synonyms have been proposed for this section; nevertheless, no study has yet evaluated the type specimens and resolved within this section the precise number of species (Sheath *et al.*, 1994b). Furthermore, species previously placed in the sections *Setacea* and *Claviformia* has been suggested by Necchi (1990) and Necchi and Entwisle (1990) to be categorized into section *Virescentia* (Sheath *et al.*, 1994a). Sheath *et al.* (1994b) studies of species within the section *Virescentia* discerned two misclassified type specimens in the section. The first type specimen *B. julianum* was noticed to possess carpogonial branches that were robustly twisted and therefore, should be categorized in the section *Contorta* (Sheath *et al.* (1994b). The second type specimen, *B. transtaganum* with two described forms, have the first form exhibiting carpogonial branches in helix shape, hence warranting classification in the section *Contorta*. The second form on the other hand, has extended carpogonial branches that arise from fascicle cells at the mid-level, hence should be classified in section *Batrachospermum* (Sheath *et al.*, 1994b). Although taxonomy had relied on certain characteristics to differentiate species in section *Virescentia*, Sheath *et al.* (1994a) observed certain characteristics that were in contrast to prior reports. For example, in all their North American populations and other specimens, Sheath *et al.* (1994b) observed restricted secondary branching, monoecious, fascicle apices with few or no singly produced hair cells, and pericentral and proximal fascicle cells formed carpogonial branches, all differing from previous findings (e.g. Sirodot, 1884; Mori, 1975; Starmach, 1977).

Similar to sections *Batrachospermum* and *Virescentia*, defining species within the section *Aristata* has also been problematic using the MSC. It has been observed that some of the characters used in distinguishing section *Aristata* from the other sections are overlapping and do not separate the sections properly. For example the species *B. breutelii* within this section is observed to have carpogonial branches with few cells, as well as cells short and similar to those observed in section *Virescentia* and section *Turfosa* (Sheath *et al.*, 1994a). Also, in section *Batrachospermum*, branches bearing carpogonia are supported by pedicels are also observed in species of section *Aristata* (Sheath *et al.*, 1994b). Kumano (1993, 2002) separated species within section *Aristata* into two subsections Macrosporum and *Aristata* with hypogenous cells at carpogonium base and without hypogenous cells at carpogonium base respectively (Figure here). Sheath *et al.* (1994a) concluded the proposed subsection by Kumano (1993) were unnecessary. Sheath *et al.* (1994a) observed variance within length and density of involucral filaments based on maturity that were found along the carpogonial branch. For example, the species *B. excelsum's* mature carpogonium is not encircled by an apparent rosette (Sheath *et al.*, 1994a) although Kumano (1993) categorized it in subsection *Macrosporum*. The main distinguishing character of section *Aristata* as proposed by Necchi and Entwisle (1990) is the presence of carpogonial branches that are straight and ca. 12 cells long. Nonetheless, Sheath *et al.* (1994a) observed in their studies that, the group that contained the species *B. cayennense* and *B. longiarticulatum* suit the view proposed by Necchi and Entwisle (1993) for this section considerably. On the other hand, the species *B. macrosporum* and *B. breutelii* are described to have shorter carpogonial branches, ca. 4-11 cells long, similar to that observed for species in section *Turfosa* (Sheath *et al.*, 1994a). The section *Aristata* species *B. breutelii* has been described in their post-fertilization gametophytes to have huge septated structures inferred to be either gemmules (Skuja, 1933), tetrasporangia (Ratnsbapathy and Kumano 1982) or of parasitic origin (Starmach, 1977). The septated structures, Sheath *et al.* (1994a) concluded, are distinct enough to justify a new section for this species, although the nature of them needs to be first confirmed. Consequently, with

all the above discrepancies within the section *Aristata* using the MSC, its taxonomy needs to be studied and resolved employing other taxonomic methods, perhaps in combination with the MSC.

Comparable to section *Batrachospermum*, *Virescentia*, *Aristata*, section *Turfosa* is defined by several morphological characters. Due to having excess characteristics either than those already used to distinguish section *Turfosa* like helically twisted carpogonium-bearing branch, some species including *B. guyanense, B. nodiflorum* and *B. toridium* are now categorized in section *Contorta* due to carpogonia twisted in a helix form (Sheath *et al.*, 1994c). As well, the species *B. cayennense, B. dimorphum, B. excelsum*, and *B. oxycladum* were previously categorized in section *Aristata* since they posses reasonably long, differentiated carpogonial branches and carposporophyte joined by pedicels (Kumano, 1990; Sheath *et al.*, 1994c). Nonetheless, currently the species *B. cayennense* is recognized as a synonymous species to *B. breutelii* under the section *Gonimopropagulum* (Kumano, 2002). Likewise, the species *B. excelsum* and *B. oxycladum* are now both recognized as synonymous to *B. macrosporum* under subsection *Macrospora*, section *Aristata* (Kumano, 2002). The species *B. dimorphum* is, however, now placed under the subsection *Ambigua*, below the section *Contorta* (Kumano, 2002). The species *B. orthostichum* has been recategorized into the section *Turfosa* from section *Setacea* by Necchi (1990) on the basis of the presence of both prostrate and erect gonimoblast filaments in the specimens. Sheath *et al.* (1993c); however, argued that *B. orthostichum* posses only erect gonimoblast filaments, and therefore fit in section *Setacea*. For species within section *Turfosa*, frequency and length of secondary fascicles, in addition to characters such as plant coloration, fascicle cell number, carpogonium and carposporangium size and shape, peripheral cortication, monosporangia absence or presence, mature or aborted carposporophytes, and spermatangia on involucral filaments are used to differentiate one species from the other (Necchi, 1990; Kumano, 2002). Nonetheless, some of these characteristics are variable and no type specimens have yet been compared to resolve the extent to which each characteristic can be used to separate species within this section (Sheath *et al.*, 1994c). Sheath *et al.* (1994c) studied species within section *Turfosa* and

observed that the type specimens of *B. bambusinum* and *B. keratophytum var. chalybeum* have been misclassified in the section *Turfosa*. *B. bambusinum* is observed to have fascicles with few cells pressed closely to the axis like observed for species in section *Setacea* (Sheath *et al.*, 1993c). On the contrary, the species *B. keratophytum var chalybeum* is observed with twisted carpogonial branches and hence proposed to be categorized in section *Contorta* (Sheath *et al.*, 1992). Nonetheless, *B. keratophytum* have previously been distinguished from the species *B. turfosum* based on whether they were carposporic or monoporic, however, studies by Müller *et al.* (1997) concluded that *B. keratophytum* and *B. turfosum* are synonymous based on small sequence divergence between ITS1 and 2 regions of the two species compared to that of other algal studies employing the same DNA region and also that plants were monosporic at different times of the year.

To differentiate the section *Hybrida* from section *Batrachospermum*, Necchi (1990) and Necchi and Entwisle (1990) added two additional features to above defined characters for section *Hybrida*; asymmetrical carpogonia, and the occurrence of both straight and curved carpogonial branches. Kumano (1993) identified three species within the section *Hybrida*, and these were *B. abilli, B. mikrogyne, and B. virgato-decaisneanum*. Nonetheless, Kumano (2002) recognizes only two species within section *Hybrida*; *B. virgato-decaisneanum* and *B. abilii* with no specified reason to omitting *B. mikrogyne*. Sheath and Vis (1995); however, described *B. mikrogyne* as a heterotypic synonym to *B. virgato-decaisneanum*. Species within section *Hybrida* are differentiated mostly on the basis of cortication density, size of trichogyne and carposporophyte, shape of whorl and trichogyne, and length and density of hairs (Flint, 1953; Reis, 1974). Morphometric data observed for *B. virgato-decaisneanum* by Sheath and Vis (1995) compared with previous studies were comparable, with one exception, longer carpogonia length in specimens from Brazil observed by Necchi (1990) compared to ones observed by Sheath and Vis (1995). This exception, however, is considered minimal and *B. virgato-decaisneanum* appears to be defined as a species in section *Hybrida* (Sheath and Vis, 1995). *B. abilii* initially was distinguished on the base of trichogyne and

whorl shape by Reis (1965b), however, the study conducted by Sheath and Vis (1995) only confirmed whorl shaped as a differentiating character. Sheath and Vis (1995) observed too much variability in trichogyne shape to be employed as character in differentiating species in section *Hybrida*. However, Sheath and Vis (1995) added whorl size as a differentiating character for the species *B. abilii*. On the contrary, the species *B. mikrogyne* was previously differentiated from *B. virgato-decaisneanum* on the basis of carpogonia size, specifically smaller size (Flint, 1953). In contrast, Sheath and Vis (1995) observed longer carpogonia in type specimens of *B. mikrogyne* than those of *B. virgato-decaisneanum*. As well, Flint (1953) noted the absence of cortication in *B. mikrogyne*, mainly on the carposporophytic plants. However; Sheath and Vis (1995) observed in abundance cortication in the carposporophytic specimens of *B. mikrogyne* they examined. Another feature employed to separate *B. mikrogyne* was unevenly large carposporophytes compared to the whorls (Flint, 1953). Although the average carposporophyte height of *B. mikrogyne* compared to whorl diameter is larger than that of *B. virgato-decaisneanum*, Sheath and Vis (1995), observed some overlap between the two species, and consequently considered them to be synonymous to each other.

The majority of the morphological characters employed to distinguish members of the Batrachospermales overlap tremendously; hence, morphometric data obtained and analyzed has, in some groups, proven ineffective in distinguishing members of the order Batrachospermales. Consequently, this calls for other taxonomic and systematic tools to resolve the various issues among the various sections. One such method is employing the molecular tool DNA barcoding to resolve the various issues at the generic and infrageneric level.

1.10 PREVIOUS MOLECULAR STUDIES ON THE ORDERS BATRACHOSPERMALES AND THOREALES

The phylogenetic and biogeographical relationships of different levels of the red algae including the order Batrachospermales and Thoreales have been examined extensively using conserved DNA sequences that code for genes such as the nuclear ribosomal small subunit (18S rRNA) gene and the large subunit of the gene encoding the chloroplast enzyme Ribulose 1,5-bisphosphate carboxylase/oxygenase (*rbc*L) (e.g. Freshwater *et al.*, 1994; Sheath and Müller, 1997; Müller *et al.*, 1998; Vis and Sheath, 1998; Vis and Entwisle 2000; Hall and Vis, 2002). The study by Vis and Sheath (1998), examined the relationship between *B. spermatoinvolucrum* and *B. gelatinosum* employing both the rRNA internal transcribed spacers (ITS) 1 and 2 sequences and the *rbc*L gene and concluded that sequence data from both genes provided different resolution capabilities with the 18S rRNA gene sequencing proving more useful in resolving internal branch relationship, while the *rbc*L gene resolved terminal branch relationships. Nonetheless, the *rbc*L gene has been used in several phylogenetic and phylogeographic studies on members of the order Batrachospermales (e.g.Vis et. al, 1998; Vis *et al.*, 2001; Hanyuda *et al.*, 2004; Vis *et al.*, 2005). While its been useful in resolving interspecific and intergeneric relationships (e.g. Freshwater *et al.*, 1995; Fredericq and Ramírez, 1996) among taxa due it high rate of mutation, the *rbc*L gene is been observed to be problematic when addressing higher level taxonomic questions due to long branch attraction (e.g. Felsenstein, 1978; Garybeal, 1998) (Freshwater *et al.*, 1999). Other molecular markers including the Inter-simple Sequence Repeat (ISSR) (e.g. Hall and Vis, 2002) have been employed in attempts to resolve either phylogenetic or biogeographical relationship among the red algae. Hall and Vis (2002) study use of ISSR to investigate genetic relationship among individuals of *B. helminthosum* among and within distant streams resulted in important genetic differentiation among the individuals studied. Nonetheless, the genetic variation among streams was substantially high such that inter-stream correlation could not be established. Hence ISSR proved limited in reflecting geographic

distance among *B. helminthosum*. Aside from the use of nuclear or plastid markers in interspecific and intraspecific studies, mitochondrial data is commonly now employed to address systematics, biogeography or population genetics questions (e.g. Zuccarello *et al.*, 2002, Chiasson *et al.*, 2003). An example of such mitochondrial marker, the noncoding region in the middle of the mitochondrial cytochrome oxidase subunit 2 and subunit 3 gene (cox2-3) has been employed in different red algae members like *B. helminthosum* and *B. macrosporum* and has proven useful in evaluating phylogeography (Chiasson *et al.*, 2003). While cox2-3 spacer has proven useful in phylogeographic studies of *B. helminthosum* and *B. macrosporum*, no studies have yet established its reliability in *B. gelatinosum*, the type species of the genus *Batrachospermum*, hence the need for a new marker to explain both the biogeography and phylogeography of this species and other members of the Rhodophytes.

1.11 BIOGEOGRAPHIC TRENDS IN NORTH AMERICAN COLLECTIONS

The red algae are considered a cosmopolitan group, and of the all described genera, an estimated 3 – 5% are observed in freshwater (Kumano, 2002; John, 2002). Freshwater algae represent organisms observed developing in inland waters and are usually photosynthetic but lack a vascular system (Sheath, 2006). The geographic distributions of freshwater red algae are believed to be impacted by factors such as temperature and water systems, continental drifts and glaciation (causing species divergence) as well as vector-assisted transport (Sheath and Cole, 1990). Vector-assisted transport could include waterfowl and muskrats transporting various kinds of algal fragments and spores via their feet, feathers and bills (Atkinson, 1980). As well, the muskrat is been observed to carry viable algae in contents of its stomach (Roscher, 1967). In North America the most substantial diversity of species have been observed in temperate and tropical latitudes with boreal regions having the fewest (Sheath and Cole, 1990). Although

observed in all biomes (except Antarctic), freshwater red algae are most common in warm-temperate and tropical climates (Orfanidis and Breeman, 1999; Kumano, 2002), being observed primarily in fast-flowing streams (Kumano, 2002). Globally, certain taxa distribution, including those of section *Contorta* and *Thorea* are observed in lowland tropical and subtropical regions (Starmach, 1977; Kumano, 1980; Sheath *et al.*, 1987). Nonetheless, *Thorea* species are observed to be abundant in temperate territories during warm months (Kremer, 1983; Entwisle and Kraft, 1984). Further, studies have reported widespread of the genus *Thorea* in Illinois, Florida, Grenada, Mexico, Ohio, Nebraska and Texa (Sheath *et al.*, 1993 and all references therein). Distribution and systmatics evaluation of the genus *Thorea* indicated scattering and isolation of populations, mostly restricted to the Costal Plain and tropical regions of North America. In Europe, however, the reach of *Thorea* is been reported to stretch north (Tomas, 1981) and in South America, Africa, and Asia have been reported to occur as localized populations (Sheath *et al.*, 1993 and references therein). In North Amercia, the Thoreaceae is been observed to occur in moderately flowing, medium to large streams. Streams with warm waters and high pH.On the contrary, member of the genus *Lemanea* are observed to occur in boreal and alpine environments (Skuja, 1938; Kumano, 1980). These geographic patterns distribution by account of Kremer (1983) are based on photosynthetic response to temperature, with *Lemanea fluviatilis* and *Batrachospermum* sp. demonstrating maximal photosynthesis at roughly 15°C. Macroalgae from North America tundra streams are observed to be distributed in accordance with ecological factors including stream velocity and freezing periods as well as ability to survive by means of how resistant their cells are (Sheath and Cole, 1992).

Several studies have employed molecular techniques to study the biogeography of algae in both marine and freshwater. This has included the use of ITS-1 and ITS-2 of rDNA by Vis and Sheath (1997), Rintoul *et al.* (1999) and Entwisle *et al.* (2000) to assess interspecies and intraspecies relationships of freshwater red algae. Similarly, the gene encoding the large subunit of the chloroplast enzyme Ribulose 1,

5-bisphosphate carboxylase/oxygenase (*rbc*L) has been employed to examine the biogeography of freshwater algae, especially that of *Bangia atropurpurea* (Müller *et al.*, 1998) and *Psilosiphon scoparium* (Entwisel *et al.* 2000). A combination of the two genes (ITS rDNA and *rbc*L) however, has been employed by Vis *et al.* (2001) to study the biogeographical trends of *B. helminthosum* in North America. Vis *et al.* (2001) observed a complex phylogeography of *B. helminthosum* with distantly located samples appearing genetically similar. Vis and Sheath (1997) study of *B. gelatinosum* over a wide geographic range in North American observed a relatively low sequence variation of the ITS region used and concluded *B. gelatinosum* to be a toxon that is morphologically variable and geographically wide spread. Like Vis and Sheath (1997), Rintoul *et al.* (1999) employed the internal transcribed spacer (ITS1 and ITS2) in addition to the 5.8S rRNA gene to study the systematic and biogeography of the order Compsopogonales (Rhodophyta). Rintoul and colleagues observed no apparent geographic trend in the Compsopogonaceae. Similarly, Entwisle *et al.* (2000) applied the ITS1-5.8S rDNA ITS2 as well as the *rbc*L to explore the biogeography of *Psilosiphon* (Batrachospermales) in Australia and New Zealand. Entwisle and colleagues observed too widespread species in their Australian clade to offer any significant hypothesis on their biogeography. Nonetheless, they observed a significant phylogeny support to indicate three *Psilosiphon scoparium* origins were prior to New Zealand separating from Gondwana over 150 million years ago (Dawkins, 2004).

1.12 DNA BARCODING: THE RATIONALE

The use of mitochondrial DNA (mtDNA) sequences in reconstructing evolutionary relationships among recently diverged animals has been in practice for over two decades (Ballard and Whitlock, 2004). Mitochondrial DNA has been established as the marker of choice for inferring species-level and generic-level relationships (Hurst and Jiggins, 2005), and hence plays a crucial role in evolutionary, biodiversity, population genetics and conservation studies (e.g. Zardoya and Meyer, 1997; Boore and Brown, 1998; Mindell *et al.*, 1998; Naylor and Brown, 1998; Rasmussen and Arnason, 1999). An average animal mitochondrial genome contains approximately 37 genes; 13 of which code for proteins, 22 for tRNA and 2 for rRNA subunits (Boore, 1999; Ballard and Whitlock, 2001) and among vertebrates the order of these genes is distinctly conserved (Brown, 1985; Boore, 1999). Mitochondrial genomes of vertebrates are usually about 16,569 base pairs (bp) long with no introns and few intergenic spacers (Broughton *et al.*, 2001; Ballard and Whitlock, 2001). The only noncoding sequence, called the control region (CR), is typically about 1100 bp long and has a function in regulating transcription and replication (Shadel and Clayton, 1997). Myriad properties of animal mtDNA have made it a preferred choice for studies of animal evolution, conservation and diversity, and these include it maternal mode of inheritance, high rate of mutation, high copy number and lack of recombination (Ballard and Whitlock, 2004).

Relying on the above unique properties of the animal mtDNA, in the past 5 years a new taxonomic method termed DNA Barcoding has been proposed as a potential method for resolving all of biodiversity. DNA Barcoding as a method employs short mtDNA genetic markers of organisms to identify species (Hebert 2003a, b; 2004). The concept of DNA barcoding is based on the assertion that most eukaryotes have mitochondria and that mitochondrial DNA (mtDNA) has a relatively fast rate of mutation (Ehara *et al.*, 2000; Hebert *et al.*, 2003; 2004a; Hurst and Jiggins, 2005). Due to its fast rate of mutation, a significant variation is seen in mtDNA sequence between species, and theoretically, a

comparatively small variance within species (Hebert *et al.*, 2003 a, b; 2004; Hurst and Jiggins, 2005). With an approximate length of 650 bp, a region of animal mitochondrial gene, cytochrome c oxidase subunit 1 (CO1) has been proposed as a potential 'barcode' (Hebert *et al.* 2003 a, b; 2004). The CO1 gene is used for a number of reasons, the first being that it lacks introns, secondly it is limited to recombination and lastly it has haploid inheritance (Hebert *et al.*, 2003a). In addition, CO1 gene has two important advantages over other mitochondrial genes. The first being the 5' end of the CO1 gene can be recovered easily from many, if not all, animal phyla because the gene is strong for universal primers due to high conservative selection pressures. Secondly, like other coding protein genes, the third-position nucleotide of the CO1 gene demonstrates high rates of base substitution that is not observed in the rRNA gene (Hebert et al, 2003a). Previous studies of protists (Bolivar et al, 2001; Sakaguchi, 2005; Litaker *et al.*, 2007; Lara *et al.*, 2007) with phylogenetic focus employed analysis of the small subunit (SSU) rDNA, and while this marker has provided valuable insight into the similarities between the major lineages of the protistan, the SSU rDNA evolves too slowly, to be employed in resolving recently derived species (de Vargas, 1997; Chen and Yu, 2000; Merzlyak *et al.*, 2001).

DNA barcoding has already been used extensively to discover and identify divergent animal taxa (Hebert et al, 2004) as well as red macroalgae (Saunders 2005, Robba *et al.* 2006; House *et al.*, 2008). In 2003, Hebert and colleagues used DNA barcoding to assign 150 newly investigated organisms to species with 100% identification success (Hebert *et al.*, 2003a). The Internal Transcribed Spacers (ITS1 and 2) was suggested by Saunders (2005) as the marker that can be used for red algae DNA barcoding as it was a tool in resolving a many species issues within the red algae, however, the ITS regions have their own shortcomings. The presence of indels within ITS 1 and 2 makes them difficult to align to those of other species because they will cause inaccurate estimation of nucleotide difference (Saunders, 2005). In a study by Robba *et al.* (2006) the use of COI in six orders of red algae indicated that one could discriminate within (intraspecific) and between (interspecific) species. Results from this study indicate

that DNA barcoding using COI works in identifying red algal species. Robba *et al.* (2006) results showed that COI gene through DNA barcoding uncovered diversity and revealed the initial stages of speciation. Witt *et al.* (2006) used DNA barcoding to examine Hyalella within the genus amphipod crustaceans. Although difficult to examine the taxonomy of this genus, Witt *et al.* (2006) through the COI nucleotide observed divergence range from 4.4% to 29.9% among the provisional species studied. DNA barcoding is very promising and current databases like Barcode of Life Data system (BOLD, http://www.barcodinglife.org) are available that aim at collecting DNA sequence from other gene regions that have been barcoded. This identification region hence could be used to study species relationships in members of the orders Thoreales, Batrachospermales and potentially assign species. Nonetheless,the use of COI in DNA barcoding, has its own.practical challenges that are relevant to mtDNA and its use in DNA barcoding to assess biodiversity and identify species. The potential drawbacks of DNA Barcoding related to mtDNA and inheritance, include recombination, maternal inheritance, heteroplasmy, reduced effective population size, unpredictable mutation rate, and a mix of genetic factors. While these drawbacks are legitimate issues for proponents of barcoding to acknowledge (e.g. mutation rate), proponents of barcoding argue some of these difficulties are often overestimated (maternal inheritance and recombination). The following explore some of the limits DNA barcoding might encounter

Maternal Inheritance

Through the cytoplasm of the oocyte, several hundreds of mitochondria and thousands of mtDNA are inherited from the female. This uniparental mode of animal mtDNA inheritance has been the persistent principle for several years and has been one of the exceptional benefits of the mtDNA, since it gives a mean to trace related lineages back through time. This property of animal mtDNA brings to light the maternal ancestry of a population avoiding the confusing effects of biparental inheritance and recombination, which are persistent in nuclear DNA (Pakendorf and Stoneking, 2005). For years it has been demonstrated that the mitochondria of sperm are destroyed in the oocyte (Manfredi *et al.*, 1997;

Shitara, 1998) through ubiquitination (Sutovsky *et al.*, 1999; 2000). Examples of these listed Korpelainen *et al.* (2004) include that of the bivalve mollusks which exhibit doubly biparental mitochondrial inheritance. Implications of different inheritance modes including defining species that ignore all process of evolution that do not affect females and a bias in interpretation of processes that affect the sexes differently. Occurrences of paternal inheritance of mitochondria are being noted across an extended range of taxa and this is increasing (Zhao *et al.*, 2004).

Recombination

Mitochondria DNA recombination in animal cells was thought to be absent or exceptionally rare (Avise, 1994; Castro *et al.*, 1998), based predominantly on the failure to observe recombinant haplotypes in studies of mtDNA variation in animal cell cultures or natural populations (Zuckerman *et al.*, 1984). The lack of recombination, as suggested by some authors (e.g. Howell, 1997; Tsaousis *et al.*, 2005), could be attributed to a possible mechanism that has evolved in the mitochondrial genome of animals to slow Muller's ratchet. Observations in current data indicate recombination in several animal species including the nematode *Meloidogyne javanica* (Lunt and Hyman, 1997), the Mediterranean mussel *Mytilus galloprovincialis* (Ladoukakis and Zouros, 2001a), blue mussel *Mytilus troussulus* (Hoarau *et al.*, 2002), and humans (Kraytsberg *et al.*, 2004) and these should be valid examples for concern for proponents of DNA barcoding. The occurrence of mitochondrial recombination would generate sequence variation by means that defy significant assumptions of DNA barcoding protocol (Rubinoff *et al.*, 2006).

Rates of Mutation

The rate of mtDNA evolution is about 5 – 10 times faster than that of the nuclear genome (Brown and Wilson, 1979) primarily due to the absence of repair enzymes to amend errors in replication and any damage to the DNA (Clayton, 1982). As a result mtDNA has a high level of mutation, ultimately leading to higher mutation rate and more divergence among the mtDNA of individuals (Wilson *et al.*, 1985). In

animals, mtDNA is observed to evolve at a rapid rate in regard to nucleotide substitution (Avis, 1994). Nonetheless, gene arrangement and genome size appear relatively consistent among species (Avis, 1994). Consequently, proponents of DNA barcoding rely on the divergence caused by mutation to delimit species. However, speciation is noted not to be driven exclusively by changes in mtDNA nor do speciation events automatically change the mtDNA haplotype (Rubinoff *et al.*, 2006). Researchers for the most part assume differentiation within a characters system indicates changes in the organism or its history (Ballard and Rand, 2005) and from the perspective of mtDNA, studies have focused on whether differentiation of the mtDNA indicates species trees or gene trees (Ballard and Rand, 2005).

Heteroplasmy

Heteroplasmy refers to the presence of more than one type of mtDNA in an individual. Considerable levels of heteroplasmy have been reported in humans (Grybowski *et al.*, 2003) insects (Nardi *et al.,* 2001; Farge *et al.,* 2002); bats (Petri *et al.,* 1996) and among other groups. Heteroplasmy may suggest that the mitochondria of an individual may denote a sampling of the alleles within a population like any other nuclear gene (Rubinoff *et al.,* 2006). Consequently, for DNA barcoding to be accurate there should be no overlap with these alleles and that of other species. While heteroplasmy maybe rare, it still possess difficulties especially on a large-scale base since these difficulties can be amplified at the global level. An example of heteroplasmy is already been observed in a study of skipper butterflies (Hebert, Penton, *et al.,* 2004).

It is agreed by several researchers that mtDNA alone is not an adequate marker to infer phylogeny at the species-level (e.g. Funk and Omland, 2003; Ballard and Whitlock, 2004; Hurst and Jiggins, 2005). However, if animal mtDNA should be employed alone as an evolutionary marker, as is done in DNA barcoding, certain tests should be included in the studies. These could include heterogeneity rate test along the length of the sequence as well as varying branches of the tree (Ballard and Rand, 2005).

Additional tests could include examining the basic assumptions and predictions of the neutral theory (i.e. a constant mutation rate, allele frequency distributions that are stationary and a link between divergence and levels of polymorphism) (Ballard and Whitlock, 2001) to enhance our ability to interpret results of animal mtDNA analyses. While there is no doubt that DNA barcoding will create some mistakes, what is uncertain is the rate of occurrence of these mistakes and whether the occurrences surpass tolerance limits (Hurst and Jiggins, 2005). Funk and Omland (2003) review of species polyphyly based on mtDNA data indicated approximately 23% of species may not, for mtDNA sequences be monophyletic, a pattern not revealed by DNA barcoding tests (Hebert *et al.*, 2003; 2004a). DNA Barcoding does not necessary aim at replacing all other taxonomic tools, but rather be part of an 'integrated' approach in resolving all of biodiversity.

DNA barcoding has been shown to be successful in identifying species in members of the red algae (e.g. Saunders, 2005; Robba *et al*, 2006; House *et al.,* 2008). Consequently, to attempt resolving taxonomic level relationship issues among members of the order Batrachospermales and ascertaining the order status of the Thoreales, DNA barcoding protocol would be employed in the present study under the following objectives:

a) Test the efficacy of the DNA barcoding protocol (COI) in resolving species relationships in the Batrachospermales and Thoreales

b) Test the usefulness of the cytochrome c oxidase subunit I (COI) in identifying interspecific and intraspecific variations in members of the Batrachospermales and Thoreales

c) Investigate ny possible geographic variation in members of the two orders and

d) Compare results of the COI gene to that of the plastid *rbc*L gene

2.0 MATERIALS AND METHODS

2.1 SAMPLE INFORMATION

Specimens employed in this study were collected from freshwater locations across three continents: North America, Europe and Africa (Table 2, Figure 3 (Represent North American Distribution)).

2.2 PCR AMPLIFICATION AND SEQUENCING

Extracts of DNA from previous studies were used with the methods of extraction outlined in those papers (Vis and Sheath, 1997; Vis and Sheath, 1998; Müller *et al.*, 2002). Other genomic DNA were extracted from materials stored at – 80^0C using a modified phenol-chloroform extraction protocol (Saunders *et al.* 1993) and for other specimens the DNeasyTM Plant Mini Kit (Qiagen Inc., Mississauga, ON, CA) was used, following the protocol outlined by the manufacturer. PCR amplification of the COI gene region were performed using the primers GazF1 5'TCAACAAATCATAAAGATATTGG 3' and GazR1, 5'ACTTCTGGATGTCCAAAAAAYCA 3' as outlined in Saunders (2005). The primer pair F160, 5' CCTCAACCAGGAGTAGATCC 3' and *rbc*L reverse 5'ACATTT GCTGTTGGAGTCTC 3' were used to amplify fragment of the 1461-bp *rbc*L gene (Vis *et al.* 1998). PCR reactions were performed in 50µL volumes for each sample. Each PCR contained 5µL of 10x PCR buffer (without $MgCl_2$), 3µL of $MgCl_2$ (25mM), 1.0 µL of dNTP (10 mM), 1.0 µL of forward primer GazF1(100mM) for COI or F160 (10mM) for *rbc*L, 1.0 µL of reverse primer GazR1(100mM) for COI or *rbc*LR (10mM) for *rbc*L, 38 µL of distilled water, 0.5 µL of Taq polymerase (Fischer), 1.0 µL of BSA and 1.0 µL of DNA template. The reactions were carried out using the Eppendorf (Eppendorf Canada, Mississauga, ON). PCR reactions were performed with 1 cycle 1 cycle of 94^0C for 2 min; 30 cycles of 94^0C for 30 s, 45^0C for 30 s, 72^0C for 1 min; 1 cycle of 72^0C for 5 min (Robba *et al.*, 2006) with annealing temperature changed to 45^0C from 500C. Amplified samples were subjected to gel electrophoresis on 1% agarose gel against the ladder Hind

III (Fisher Scientific Canada, Ottawa, ON). Consequently, QiaQuick PCR Amplification System (Qiagen Inc. Mississauga, ON, CA) was used to purify all positive PCR products result and sequencing was performed using the ABI 3130XL capillary sequencer (Applied Biosystems Canada, Streetsville, ON, CA). For sequencing, GazR1 was used for majority of the COI products. For products of *rbc*L, both the forward F160 and reverse *rbc*LR were used as well as the internal primer R897, 5' CGTGAGTATGTTGAATTACCTGC 3' for results missing internal base pairs.

Table 2: Collection Information for Order Batrachospermales and Thoreales Samples used in the Present Study

Taxa #	Taxon	Taxon ID	Collection Information	COI	*rbc*L
	B. ambiguum Montagne	AUS	Australia		•
	B. ambiguum	CR27	Los Alturus, Costa Rica	•	
	B. ambiguum	CR22	Costa Rica	•	
	B. ambiguum	CR25	Rio Coton, Costa Rica, Coll.: R. Sheath & K. Müller, 19.Feb.98		•
	B. ambiguum	BLZ2a	Little Vaqueros Creek at Rt. A-10, Pine Ridge, Belize, Coll.: R. Sheath, 01.Jan.00		•
	B. ambiguum	BLZ13a	Belize	•	
	B. androinvolucrum Vis et Cole	BC126	Last Shoe Ck at Rt. 4, 8 km east of Uclulet, B.C., Coll.: R. Sheath, 15.Aug.89		•
	B. atrum Harvey	BI13	Blue Hole near Stanford Dingley, UK, Coll.: R. Sheath, 24.May.96	•	•
	B. atrum	IR20	Rt. 261 7 km north of Ardara, Ireland, R. Sheath, 13.Jul.97		•
	B. atrum	SCO14	Scotland	•	
	B. boryanum Sirodot	ONBSC2a	Blue Springs Creek, Rockwood, Ontario, Canada, Coll.: R. Sheath, 26.Sep.96		•
	B. boryanum	ONBSC2d	Blue Springs Creek, Rockwood, Ontario, CAN	•	•
	B. boryanum	FL60	Florida, USA	•	
	B. bruetelii Rabenhorst		Steenboks River, Cape of Good Hope, South Africa, Coll.: J. Bolton, 24.Nov.93	•	•
	B. confusum Hassal	ON	Ontario, CAN	•	
	B. gelatinosum De Candolle	AT9	Small river at Newhaus, Austria, Coll.: R. Sheath, 21.Jun.98		•
	B. gelatinosum	NF211	Newfoundland, CAN	•	
	B. gelatinosum	NH40	New Hampshire, USA	•	
	B. gelatinosum	NS28	Cheticamp River, Cheticamp, Nova Scotia, Canada	•	
	B. gelatinosum	LAB10	Labrador, CAN	•	
	B. gelatinosum	ON11	Blue Springs Creek at Guelph Line, ***5*** km SW of Rockwood, Ontario, CAN	•	
	B. gelatinosum	IRI8	Ireland	•	
	B. gelatinosum	Vancouver Island 1	British Columbia, Canada	•	
	B. gelatinosum	Vancouver Island 3	British Columbia, Canada	•	
	B. globosporum Israelson	TX9	Iandau Park, New Brunfels, Texas, USA	•	
	B. globosporum	AZ10a	Montezuma well outlet canal, Arizona, USA, Coll.: R. Sheath, 01.Dec.93	•	•
	B. globosporum	ONFR	French River, Ontario, Canada, Coll.: G. Lemon, 29.Jun.97		•
	B. helminthosum Bory	Oak Ridge	Tennessee, USA	•	

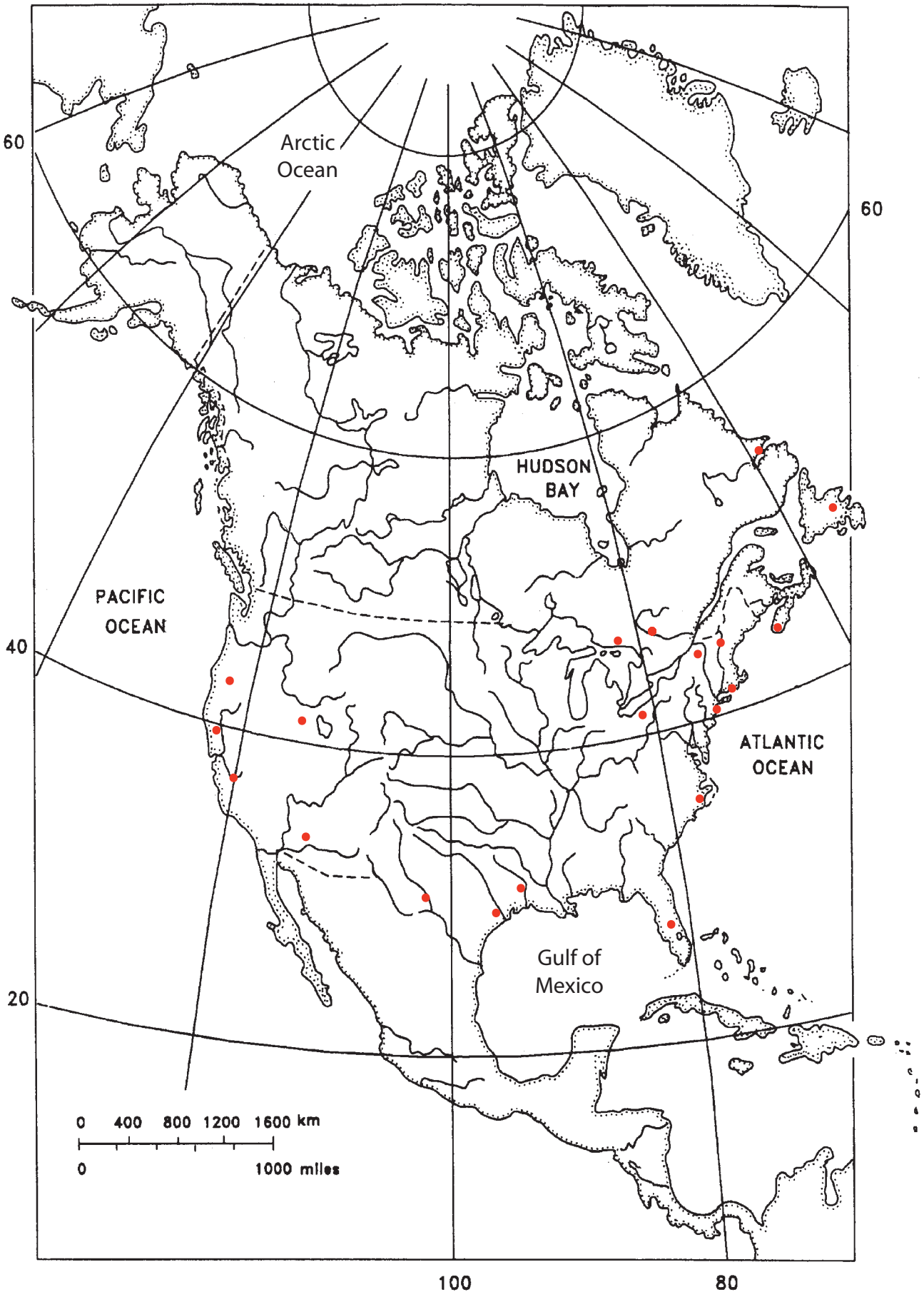
Taxa #	Taxon	Taxon ID	Collection Information	COI	*rbc*L
	B. helminthosum	RI1	Chipuxet River at Taylor's Landing, North Kingston, Rhode Island, U.S.A**.**	•	
	B. helminthosum	ONFR	Flat Rock, Ontario, Canada		•
	B. involutum Sheath et Vis	San Marcos TX	San Marcos, Texas, USA	•	
	B. involutum	TX35	Frio River, Texas, USA, TX36, Coll.: A. Sherwood, 31.May.97		•
	B. turfosum Bory	JPC	Newfoundland	•	
	B. turfosum	Moscow B	Ontario	•	•
	B. turfosum	AT21	Biedringer Platte, Austria, AT21, Coll.: R. Sheath, 01.Jul.98		•
	B. turfosum	MINN	Minneapolis	•	
	B. turfosum	MS3a	Mississippi , USA	•	
	B. louisianae Skuja	TX36	Baverlein Ck at Rt. 337, Texas, Coll.: R. Sheath, 3.May.97	•	•
	B. louisianae	M57	Rt. 26 near Wolf R., Mississippi, Coll.: R. Sheath, 3.Jul.93		•
	B. macrosporum Montagne	MS1a	Rt. 57, 0.5km n. of Leaf, Mississippi, Coll. : R. Sheath, 27.Nov.93	•	
	B. macrosporum	MS9	Mississippi, USA	•	
	B. procarpum Skuja	LA21	Bogue Lusa Creek, Louisiana, USA, Coll. : K. Müller & D.Couture, 24.Jun.00		•
	B. procarpum	LA22	Louisiana, USA	•	
	B. procarpum	LA27	Louisiana, USA	•	
	Batrachospermum sp.	BI13	Blue Pool Stanford-Dingley, Bradfield, British Isles	•	•
	Batrachospermum sp. 1	Kenya 7	Lake Victoria, Kenya	•	
	Batrachospermum sp. 2	Kenya 7	Lake Victoria, Kenya	•	
	Batrachospermum sp. 4	Kenya 9	Lake Victoria, Kenya	•	•
	Batrachospermum sp. 5	Kenya 9	Lake Victoria, Kenya	•	
	Batrachospermum sp. 6	Kenya 9	Lake Victoria, Kenya	•	
	Batrachospermum sp. 1	Kenya 11	Lake Victoria, Kenya	•	•
	Batrachospermum sp.	Kenya 9	Lake Victoria, Kenya	•	
	Batrachospermum sp.	Kenya 12	Lake Victoria, Kenya	•	•
	Batrachospermum sp.	Kenya 14	Lake Victoria, Kenya	•	•
	Batrachospermum sp.	NRC	Nova Scotia, Canada	•	•
	Batrachospermum sp.	NS CB	Nova Scotia, Canada	•	

Taxa #	Taxon	Taxon ID	Collection Information	COI	*rbc*L
	B. virgatum helminthosum Bory	RI40	Rhode Island, USA	•	
	B. virgatum	CT24	Connecticut, USA	•	•
	Sirodotia sp.	RI1	Chipuxet River at Taylor's Landing, North Kingston, Rhode Island, U.S.A.	•	
	Sirodotia sp.	RI24	Stream at Town Farm Rd., Coventry, Rhode Island, U.S.A.	•	•
	S. huillensis Skuja	TX7	San Marcos River, Peppers, Texas, USA	•	
	S. suecica Kylin	SWE13	Sweden	•	
	Tuomeya sp.	Little Rock	Little Rock, North Carolina, USA	•	
	Tuomeya sp.	Barton NC	Barton, North Carolina, USA	•	
	Tuomeya sp.	NH15	New Hampshire, USA	•	
	Thorea sp.	TX SM	Texas, USA	•	
	Thorea sp.	NY	New York, USA	•	•
	Thorea ramosa			•	•
	Thorea okoidai			•	
	Thorea violacea Bory			•	
	Paralemanea sp.	Kenya 12	Kenya	•	•
	Lemanea fucina	AT11	Austria		•
	Lemanea fucina	AT12	Austria		•
	Lemanea sp.	BC52	British Columbia, Canada		•
	Lemanea fluviatilis C. Agardh	BC76C	British Columbia, Canada		•
	Lemanea fluviatilis	BC76	Halfmoon Bay Creek at the intersection of Redrooffs Rd and O'Brien Rd, 0.2 km west of Sunshine Coast Highway, British Columbia, Canada		•
	Lemanea fucina	BI2	British Isles		•
	Lemanea fucina	BI12	British Isles		•
	Lemanea sp.	BI15	British Isles		•
	Lemanea fluviatilis	BI16	British Isles		•
	Lemanea sp.	BI17	British Isles	•	•
	Lemanea fluviatilis	FRON	Flat Rock, Ontario, Canada		•
	Lemanea borealis Atkinson	FRNF	French River, Newfoundland, Canada		•
	Lemanea fluviatilis	IR3	Ireland		•

Taxa #	Taxon	Taxon ID	Collection Information	COI	*rbc*L
	L. fucina var. parva Vis et Sheath	NH5	Rt. 16, 1 km s. of Milton, New Hampshire, USA. Coll.: M. Vis. 26.May.89		•
	Lemanea fucina	NH15	New Hampshire, USA		•
	Lemanea fluviatilis	NS32	Nova Scotia, USA		•
	Lemanea fluviatilis	OR110a	Oregon, USA		•
	Lemanea fluviatilis	OR115	Oregon, USA		•
	Lemanea fluviatilis	OR117	Oregon, USA		•
	Lemanea sp.	OR118	Oregon, USA	•	
	Lemanea fluviatilis	OR122	Oregon, USA		•
	Lemanea fucina	SCO1	Scotland		•
	Lemanea species	SCO6	Scotland		•
	Lemanea fluviatilis	SWE4	Sweden		•
	Lemanea fluviatilis	SWE8	Sweden		•
	Lemanea sp.	SWE11	Sweden	•	•
	Lemanea fucina	SWE12	Sweden		•
	Lemanea fluviatilis	SWE12	Sweden		•
	Lemanea fluviatilis	SWE13	Sweden		•
	Lemanea fucina	SWE21	Sweden		•
	Lemanea fluviatilis	WAL7	Wales, UK		•
	Lemanea fluviatilis	WAL12	Wales, UK		•
	Paralemanea catenata Vis et Sheath	IDAHO	Idaho, USA	•	
	Paralemanea sp.	Little Rock	Little Rock, North Carolina, USA		•
	Paralemanea annulata Vis et Sheath	NC	North Carolina, USA		•
	Paralemanea annulata	OHIO	Ohio, USA		•
	Paralemanea sp.	CA14	California, USA	•	•
	Paralemanea sp.	CA16	California, USA	•	•
	Paralemanea sp.	CA17	California, USA	•	
	Paralemanea catenata	CA24	Napa River at Calistoga, CA, USA. Coll. R. Sheath. 28.Jun.93	•	•
	Paralemanea sp.	CA26	California, USA	•	•

Figure 3: Geographic Distribution of North America Specimen Employed in Study

Red dots on the figure approximate North America rivers and lakes from the following states and provinces: Arizona (AZ), British Columibia (BC), California (CA), Connecticut (CT), Florida (FL), Idaho (ID), Louisiana (LA), Mississippi (MS), North Carolina (NC), New Hampshire (NH), Newfoundland and Labrador (NL), Nova Soctia (NS), New York (NY), Ohio (OH), Ontario (ON), Oregon (OR), – Rhode Island (RI), Tennessee (TN), and Texas (TX) where specimens where collected for the present study. Notably, collection of specimens was along the coastal plains.



0 800 1600 km
0 1000 miles

Table 3: Accession Numbers of COI gene sequences used from GenBank

Taxon	Citation	Accession Number
B. ambiguum	House *et al.* 2008	EU095970
B. ambiguum	Sherwood *et al.* 2008	EU636723
B. ambiguum	Sherwood *et al.* 2008	EU636724
B. gelatinosum	Sherwood *et al.* 2008	EU636743
B. gelatinosum	Sherwood *et al.* 2008	EU636744
B. helminthosum	House *et al.* 2008	EU073844
B. helminthosum	House *et al.* 2008	EU073845
B. helminthosum	House *et al.* 2008	EU073847
B. helminthosum	House *et al.* 2008	EU073848
B. heterocorticum	Sherwood *et al.* 2008	EU636740
B. intortum	Sherwood *et al.* 2008	EU636717
B. macrosporum	Sherwood *et al.* 2008	EU636747
B. macrosporum	Sherwood *et al.* 2008	EU636748
B. macrosporum	Sherwood *et al.* 2008	EU636756
B. macrosporum	Sherwood *et al.* 2008	EU636759
Batrachospermum sp.	Sherwood *et al.* 2008	EU636741
B. turfosum	House *et al.* 2008	EU095972
B. turfosum	House *et al.* 2008	EU636745
B. turfosum	Sherwood *et al.* 2008	EU636746
Sirodotia suecica	Sherwood *et al.* 2008	EU636737
Sirodotia sp.	Sherwood *et al.* 2008	EU636738
Sirodotia huillensis	Sherwood *et al.* 2008	EU636739
Neodilsea sp.	Saunders 2005	AY970617

Table 4: Accession Numbers of *rbc*L gene sequences used from GenBank

Taxon	Citation	Accession Number
B. ambiguum	Vis and Entwisle 2001	AF209988
B. ambiguum	Vis *et al.* (unpublished)	AY423390
B. atrum	Vis and Entwisle 2001	AF209979
B. boryanum	Vis *et al.* 1998	AF029140
B. gelatinosum	Vis *et al.* 1998	AF029141
B. gelatinosum	Yang and Boo (unpublished)	DQ787560
B. gelatinosum	Stewart 2007	EF375888
B. gelatinosum	Vis and Stewart (unpublished)	DQ393134
B. helminthosum	Hanyuda *et al.* 2004	AB114645
B. helminthosum	Hanyuda *et al.* 2004	AB114644
B. intortum	Vis *et al.* (unpublished)	AY423397
B. involutum	Vis *et al.* 1998	AF029143
B. louisianae	Vis *et al.* 1998	AF029144
B. macrosporum	Chiasson *et al.* (unpublished)	AY460203
B. macrosporum	Chiasson *et al.* (unpublished)	AY423419
Batrachospermum sp.	Freshwater *et al.* 1994	U04035
B. turfosum	Vis *et al.* (unpublished)	AY423407
B. turfosum	Vis *et al.* 1998	AF029147
Lemanea sp.	Braly *et al.* (unpublished)	DQ523257
L. fucina var. parva	Vis *et al.* 1998	AF029151
L. fluviatilis	Vis *et al.* 1998	AF029150
L. borealis	Vis *et al.* 1998	AF029149
P. catenata	Vis *et al.* 1998	AF029154
P. annulata	Vis *et al.* 2007	DQ449029
P. annulata	Vis *et al.* 1998	AF029153
P. palmate	Kato 2008	AB275866
P. marginicrassa	Kato 2008	AB275867
P. palmate	Freshwater *et al.* 1994	U04186
S. huillensis	Vis and Sheath (unpublished)	AF126414
S. huillensis	Vis and Sheath (unpublished)	AF126410
S. huillensis	Vis *et al.* 1998	AF029157
T. violacea	Hanyuda *et al.* (unpublished)	AB159657
T. hispida	Hanyuda *et al.* (unpublished)	AB159653
T. hispida	Hanyuda *et al.* (unpublished)	AB159652
T. violacea	Müller *et al.* 2002	AF506271
T. hispida	Müller *et al.* 2002	AF506270
T. violacea	Müller *et al.* 2002	AF506269
T. americana	Vis *et al.* 1998	AF029159
T. americana	Braly *et al.* (unpublished)	DQ523253

2.3 PHYLOGENETIC ANALYSES OF COI AND *RBC*L

Sequences obtained for samples from Table 1 were used in addition to sequence data of samples of Rhodophyta taxa from GenBank (Table 3 and Table 4). The collected data set in conjunction with unweighted pair group method with arithmetic mean (UPGMA) was used to construct a tree for COI. The UPGMA was determined using Kimura-2-parameter distances (pairwise deletion) using MEGA 3.1 (Kumar *et al.*, 2005). For phylogenetic analyses, sequences were aligned using MUSCLE v.3.6 (Edgar 2004) and ModelTest v.3.7 (Posada and Crandall 1998) was used to determine the model of nucleotide evolution. A neigbour-joining (Saitou and Nei, 1987) tree was contructed using General Time-Reversible model of nucleotide evolution and bootstrap resampling (1000 replicates) using PAUP* v4b10 (Swofford 2003). Maximum likelihood (ML) analysis was performed with 10 replicates of a heuristic search with a random addition of sequences. Parsimony trees were generated using a heuristic search under the constraints of random sequence addition (1000 replications), steepest descent, and tree bisection-reconnection (TBR) branch swapping according to PAUP* v4b10 (Swofford 2003). Bayesian posterior probability support for tree nodes was also calculated using MrBayes (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). For analysis of the COI, trees were outgrouped using *Neosildea* sp. and for *rbc*L gene data, trees using *Palmaria* sp., both closely related taxa to the groups studied.

3.0 RESULTS

3.1 COI GENE SEQUENCE ANALYSES

Sequences of the mitochondrial gene encoding the cytochrome oxidase subunit 1 (COI) were obtained for 67 samples (Table 2) from the two orders Batrachospermale and Thoreales and 28 species of freshwater red algae. In addition, 23 sequences from GenBank were included in the COI sequence analyses (Table 3). The COI spacer region is approximately 650 base pairs (bp) long; however, the sequences obtained in this study varied from about 590 to 705 bp and resulted in a final alignment length of 642 bp (including alignment gaps). The entire data set included members of the genera *Batrachospermum, Tuomeya*, *Sirodotia*, *Lemanea* and *Paralemanea* in the order Batrachospermales and genus *Thorea* of the order Thoreales. Where possible, new DNA extractions of original plant material were undertaken, however, amplification of the genes of interest were conducted on older extractions for which there was no original material. This resulted in amplification difficulties, which could be attributed to the fact that they were stored in water and therefore the genomic DNA may have degraded over time, as well as low concentration of DNA.

Figure 4 represents the UPGMA tree derived from COI sequence data. This figure highlights both the intraspecific and interspecific genetic variation of the orders Batrachospermales and Thoreales studied, demonstrating that the COI marker is viable in discriminating between and within species. Overall, twelve distinct clusters are observed in the UPGMA tree. The order Batrachospermales is represented by seven sections (*Batrachospermum, Setacea, Virescentia, Turfosa, Contorta, Gonimopropagulum* and *Aristata*) and two genera (*Tuomeya* and *Sirodotia*). Five samples of the Thoreaceae were amplified and are observed as a monophyletic cluster in Clade D (Figure 4). At the ordinal level, grouping of both Batrachospermales and Thoreales is observed, nonetheless, the order

Thoreales appears to be a clade within the order Batrachospermales which is in contrast to the recent separation of the Thoreales from the order Batrachospermales as its own order by Müller *et al.* (2002). Intraspecific variation analysis over the data set resulted in an average variation between the two orders Batrachospermales and Thoreales of 102 bp (15.9%) with pairwise nucleotide differences ranging from 0 to 149 bp for members of order Batrachospermales. The only species represented by a number of samples that do not form a monophyletic group is *B. atrum*, with *B. atrum* BI13 grouping with the family Lemaneaceae; nonetheless, they appear close together on the phenogram. The species *B. gelatinosum* is represented by the most number of sequences (9 samples from North America and 1 from Ireland) in this data set and vary intraspecifically by 0 to 15 bp (2.3%). Geographic trend is not evident in the phenogram and would require additional samples from Ireland and Europe in general in order to address the pairwise nucleotide difference between the two continents. Members of the order Thoreales differed by an average of 81 bp and are represented by Clade D (Figure 4)**.** As expected**,** interspecific variation is higher than intraspecific variation. For example, the section *Contorta* (Clade A, Figure 4) contains 6 different species in which the interspecific variation ranges from 58 bp (9.03%) (between *B. procarpum* and *B. intortum*) to 78 bp (12.15%) (between *B. ambiguum* and *B. globosporum*). For the genus *Batrachospermum*, at the section level all seven sections can be clearly identified with 4 being distinctly monophyletic with the exception of section *Contorta* and section *Gonimoopropagulum* since the monotypic species *B. bruetelii* of section *Gonimoopropagulum* group within the section *Contorta*. At the generic level, distinct clades of *Tuomeya* (Clade J, Figure 4), *Sirodotia* (Clade L, Figure 4), *Lemanea* (Clade H, Figure 4), and *Paralemanea* (Clade I, Figure 4) are observed.

A Neighbor-Joining (NJ) tree analysis (distance=gtr, rates=gamma, shape=0.4894 pinvar=0.3348) of the COI gene is depicted in Figure 5. Parsimony analysis of 334 (52.02%) parsimonious-informative characters resulted in the most parsimonious tree which is shown in Figure 6, with a tree length of 2170, Consistency Index (CI) = 0.3255 and Retention Index (RI) = 0.7842. The maximum likelihood (ML) tree (Figure 7) is similar in topology to that of the ones generated by Bayesian analyses, hence the Bayesian

consensus values are shown on the ML tree (Figure 7)(above branches). Comparison of the topologies of all three phylogenetic methods (NJ, MP, and ML) trees based on the nucleotide sequence data of COI revealed distinct patterns (Figures 5, 6 and 7). In the NJ analysis, 12 clusters are observed (Figure 5). The cluster Clade E (Figure 5) represents members of the section *Aristata* made up solely of *B. macrosporum* and two *Batrachospermum* haplotypes (NSCB and NRC) that by percent nucleotide sequence divergence and bootstrap support (BS)(86%) appear to belong to the section *Aristata*. A solid well-supported cluster of *B. macrosporum* (EU636759, MS1a, MS9) (BS 86%) is seen in Clade E, Figure 5. Nonetheless, the other two remaining collections of *B. macrosporum* (EU636747, EU636748) also well-supported (BS 100%) appear to be different from *B. macrosporum* (EU636759, MS1a, MS9)(BS 86%) and *Batrachospermum* sp. (NSCB and NRC).If the isolates *Batrachospermum* sp. (NSCB and NRC) be identified as anything other than *B. macrosporum*, then it can be asserted that *B. macrosporum* is clearly paraphyletic. Nonetheless this assertion is not as plausible since only a single speceies *B. macrosporum* is recognized under section *Aristata*. Nonetheless, should the *Batrachospermum* isolates (NSCB and NRC) be identified as *B. macrosporum*, then it appears Clade E (Figure 5), contain three distinct groups of *B. macrosporum*. Clades C (Figure 5) is made up B. turfosum and denote the section *Turfosa*, a well-supported clade (NJ, 100% BS; MP, 100% BS; ML, 0.96). Clade A (Figures 5, 6, and 7) is a cluster of *B. ambiguum, B. intortum, B. procarpum, B. globosporum, B. confusum, B. louisianae* and two *Batrachospermum* haplotypes (*Batrachospermum* sp. 1 Kenya 7, *Batrachospermum* sp. 2 Kenya 7) and represent the section *Contorta*. Specimen within this section Clade A (Figures 5, 6, and 7) represent samples spanning the three continents Africa, North America and Europe, and appear genetically diverge even for the same species, except for the two Kenya samples (sp. 1 Kenya 7, sp. 2 Kenya 7; Clade A, Figures 5 and 6) which group closely together, suggesting these two localized samples are genetically identical. Clade A in ML (Figure 7); however, show some *B. ambiguum* specimens from distant geographic areas grouping closely together and well-supported (1.00) like that observed for the two Kenya samples (sp. 1 Kenya 7, sp. 2 Kenya 7). Even so, no apparent biogeographic trends are evident within section *Contorta* for NJ, MP and ML (Clade A, Figures 5, 6, and 7 respectively). In addition,

section *Contorta* is not monophyletic in all three methods includes within it the monotypic section *Gonimopropagulum* (*B. breutelii*). In the NJ tree, *B. breutelii* is shown to form a sister taxon with *B. procarpum*, both of which form a sister taxa with *B. intortum* with strong support (100% BS). In both MP and ML trees however, *B. breutelii* is observed to form a sister taxon with *B. intortum* also with strong to moderate support (MP, 100% BS; ML, 0.72).

The cluster, Clade G and K represent that of section *Batrachospermum*. Members of this section are clearly cryptic since they are characterized by two clades (Clades G and K) that are slightly distant from each other on the phylogenies and with relationships that are generally congruent with the phylogeny of all three methods (NJ, MP and ML) with strong support (Clade G and Clade K: NJ, 100% BS; MP, 100% BS; ML, 1.00). Samples of the type species of section *Batrachospermum*, *B. gelatinosum* occurred primarily in Clade K (Figures 5, 6 and 7). Samples of this clade range from wide geographic areas spanning North America (Canada) and Europe (Ireland) with the Canadian specimens grouping closely together and forming a sister taxon with the specimen from Ireland. Nonetheless, more samples from Canada and Europe would be needed to ascertain any biogeographic trends. Clade G (Figures 5, 6, and 7) is made up of *B. heterocorticum, B. boryanum* and *B.involutum* mostly from North America and like Clade K (Figures 5, 6, and 7), more specimen would be need to establish any biogeographic patterns. Further, Clade G (Figures 5, 6, and 7) forms a well-supported (NJ, 98% BS; MP, 89% BS; ML, 1.00) sister taxon with section *Setacea* (Clade F, Figures 5, 6, and 7). Section *Setacea* is made up of *B. atrum* and two *Batrachosperum* haploptypes (5 Kenya 9 and BI13) with strong support (NJ, 91% BS; MP, 95% BS; ML, 1.00). Like section *Setacea*, Clade B (Figures 5, 6, and 7) representing section *Virescentia* is well-supported (NJ, 100% BS; MP, 100% BS; ML, 1.00). *Batrachospermum helminthosum*, the predominant species of section *Virescentia* emerge as paraphyletic since it forms three subgroups that are well-support (NJ, 100%/66%/96% BS; MP, 100%/99%/96% BS; ML, 1.00/0.91/1.00) and appear distinct from each other. At the generic level, five distinct genera (*Sirodotia, Tuomeya, Lemanea, Paralemanea* and *Thorea*) are depicted in Figures 5, 6 and 7. In the NJ tree, genus *Paralemanea* is shown to form a

well-supported (91% BS) polytomy with *B. atrum* BI13 and the clade that branches off to form the genera *Lemanea, Tuomeya, Sirodotia* and section *Batrachosperum*. This polytomy is however not maintained in the MP or ML phylogenies. The genus *Paralemanea* (Clade I, Figures 5, 6, and 7) is well-supported (NJ, 100% BS; MP, 100% BS; ML, 1.00) with a single genetically distinct specimen of *Paralemanea* from Kenya occurring as a sister taxon to the rest of *Paralemanea* specimens, also with high support (NJ, 100% BS; MP, 100% BS; ML, 1.00). The genus *Lemanea* (Clade H, Figures 5, 6, and 7) is observed as sister taxa represented by two species, one from Oregon (OR118) and the other from Sweden (SWE11), with moderate support (NJ, 69% BS; MP,55% BS; ML, 1.00). Unlike the genus *Lemanea*, the genus *Tuomeya* (Clade J, Figures 5, 6 and 7) is very well supported (NJ, 100% BS; MP, 99% BS; ML, 1.00) and made of specimen soley from North America (USA). Both genera *Lemanea* and *Tuomeya* would require additional specimen from North America and Europe to deduce any biogeographic patterns. The Thoreaceae, represented by the genus *Thorea* (Clade D, Figures 5, 6 and 7) as observed in all phylogenies is incongruent with recent findings (e.g Müller *et al.*, 2002; present study) of this family and order. The Thoreaceae in all phylogenies (Clade D, Figures 5, 6 and 7) comprise *T. ramos, T. okoidai, T. violacea* and *Thorea* sp. (NY and TX SM). In the NJ, MP and ML analysis Clade D (Figures 5, 6, and 7) are shown to form a well-supported (NJ, 85% BS; MP, ML, 0.84) sister taxon with section *Aristata* (Clade E, Figure 5). The final clade, Clade B in Figures 5, 6 and 7 represent the section *Virescentia* comprising the species *B. helminthosum* and *B. virgatum*. While same species within this section appear to be genetically distant, their cluster is very well supported (NJ, 100% BS; MP, 100 % BS; ML, 1.00). Overall, despite variations in bootstrap support values, there was concordance in the topologies of the phylogenetic trees obtained for the COI data set, although may not be compatible with the *rbc*L gene sequence data.

Figure 4: UPGMA Tree Derived from COI Sequence Data

Phenogram identifies 12 distinct clusters of members of Batrachospermales and Thoreales, reflecting the phenotypic similarities between sequences.

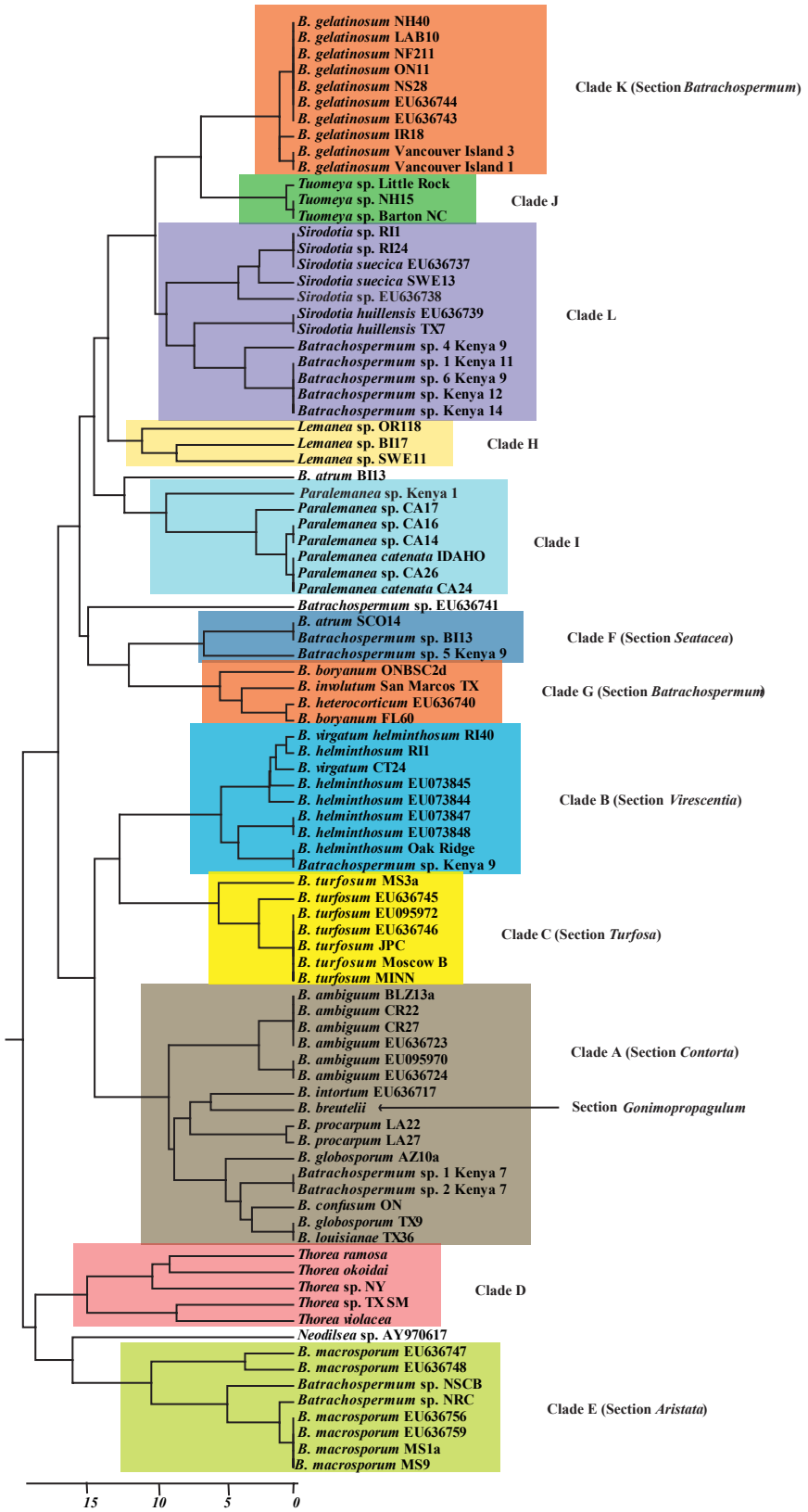

B. gelatinosum NH40
B. gelatinosum LAB10
B. gelatinosum NF211
B. gelatinosum ON11
B. gelatinosum NS28
B. gelatinosum EU636744
B. gelatinosum EU636743
B. gelatinosum IR18
B. gelatinosum Vancouver Island 3
B. gelatinosum Vancouver Island 1
Clade K (Section Batrachospermum)
Tuomeya sp. Little Rock
Tuomeya sp. NH15
Tuomeya sp. Barton NC
Clade J
Sirodotia sp. RI1
Sirodotia sp. RI24
Sirodotia suecica EU636737
Sirodotia suecica SWE13
Sirodotia sp. EU636738
Sirodotia huillensis EU636739
Sirodotia huillensis TX7
Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. 1 Kenya 11
Batrachospermum sp. 6 Kenya 9
Batrachospermum sp. Kenya 12
Batrachospermum sp. Kenya 14
Clade L
Lemanea sp. OR118
Lemanea sp. BI17
Lemanea sp. SWE11
Clade H
B. atrum BI13
Paralemanea sp. Kenya 1
Paralemanea sp. CA17
Paralemanea sp. CA16
Paralemanea sp. CA14
Paralemanea catenata IDAHO
Paralemanea sp. CA26
Paralemanea catenata CA24
Clade I
Batrachospermum sp. EU636741
B. atrum SCO14
Batrachospermum sp. BI13
Batrachospermum sp. 5 Kenya 9
Clade F (Section Seatacea)
B. boryanum ONBSC2d
B. involutum San Marcos TX
B. heterocorticum EU636740
B. boryanum FL60
Clade G (Section Batrachospermum)
B. virgatum helminthosum RI40
B. helminthosum RI1
B. virgatum CT24
B. helminthosum EU073845
B. helminthosum EU073844
B. helminthosum EU073847
B. helminthosum EU073848
B. helminthosum Oak Ridge
Batrachospermum sp. Kenya 9
Clade B (Section Virescentia)
B. turfosum MS3a
B. turfosum EU636745
B. turfosum EU095972
B. turfosum EU636746
B. turfosum JPC
B. turfosum Moscow B
B. turfosum MINN
Clade C (Section Turfosa)
B. ambiguum BLZ13a
B. ambiguum CR22
B. ambiguum CR27
B. ambiguum EU636723
B. ambiguum EU095970
B. ambiguum EU636724
B. intortum EU636717
B. breutelii
B. procarpum LA22
B. procarpum LA27
B. globosporum AZ10a
Batrachospermum sp. 1 Kenya 7
Batrachospermum sp. 2 Kenya 7
B. confusum ON
B. globosporum TX9
B. louisianae TX36
Clade A (Section Contorta)
Section Gonimopropagulum
Thorea ramosa
Thorea okoidai
Thorea sp. NY
Thorea sp. TX SM
Thorea violacea
Clade D
Neodilsea sp. AY970617
B. macrosporum EU636747
B. macrosporum EU636748
Batrachospermum sp. NSCB
Batrachospermum sp. NRC
B. macrosporum EU636756
B. macrosporum EU636759
B. macrosporum MS1a
B. macrosporum MS9
Clade E (Section Aristata)
15
10
5
0

Figure 5: Neighbor Joining (NJ) Tree derived from COI Sequences Data

Sequences were aligned using MUSCLE (ver.3.6) (Edgar, 2004) and the tree generated using to PAUP* v4b10 (Swofford 2003) over 1000 replicates. The scale indicates percent nucleotide sequence divergence. Nodes with <50% bootstrap support are not labelled. The tree is outgrouped using the COI gene nucleotide sequence of *Neodilsea* sp.

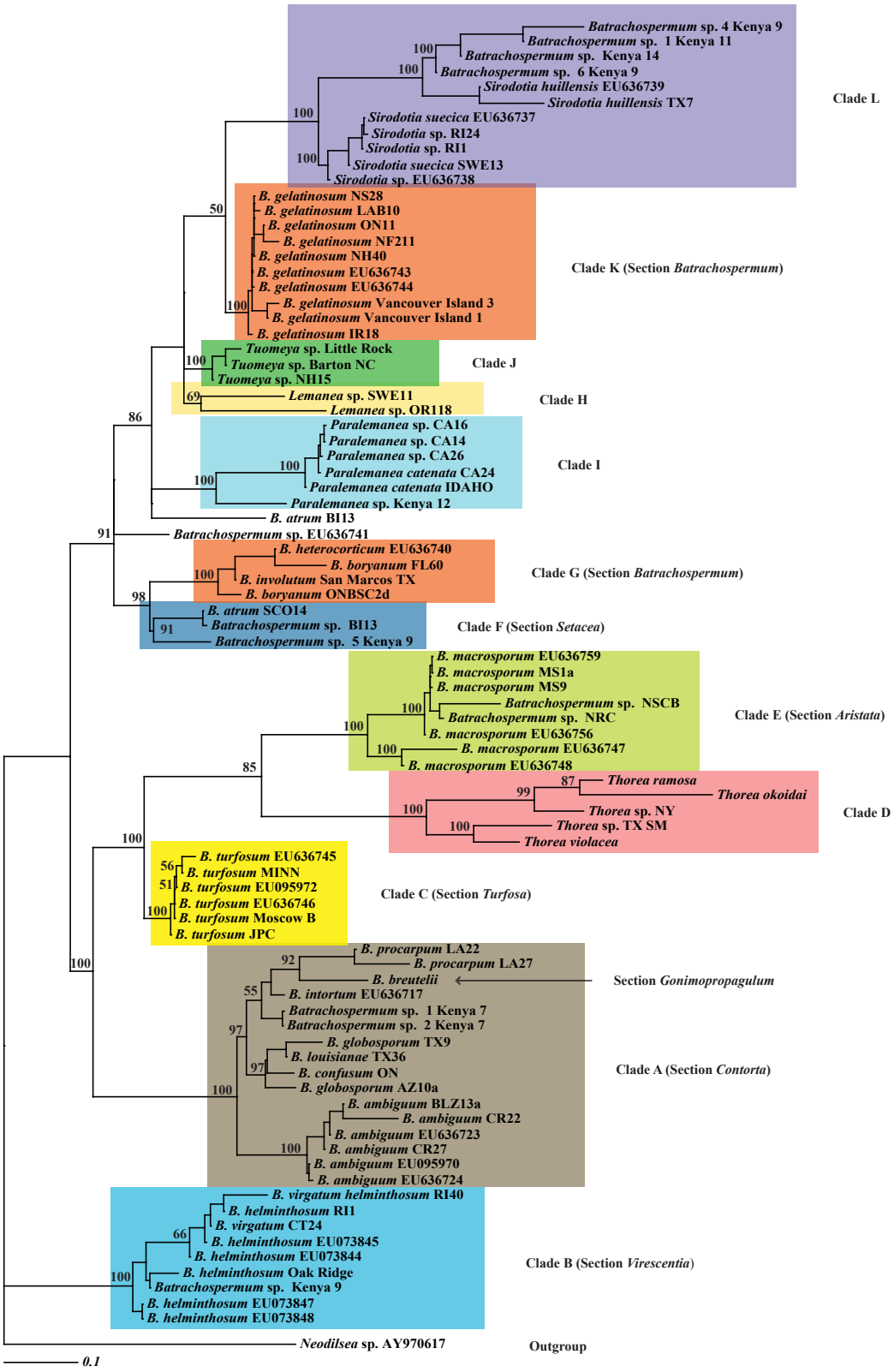

Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. 1 Kenya 11
Batrachospermum sp. Kenya 14
Batrachospermum sp. 6 Kenya 9
Sirodotia huillensis EU636739
Sirodotia huillensis TX7
Sirodotia suecica EU636737
Sirodotia sp. RI24
Sirodotia sp. RI1
Sirodotia suecica SWE13
Sirodotia sp. EU636738
Clade L
B. gelatinosum NS28
B. gelatinosum LAB10
B. gelatinosum ON11
B. gelatinosum NF211
B. gelatinosum NH40
B. gelatinosum EU636743
B. gelatinosum EU636744
B. gelatinosum Vancouver Island 3
B. gelatinosum Vancouver Island 1
B. gelatinosum IR18
Clade K (Section Batrachospermum)
Tuomeya sp. Little Rock
Tuomeya sp. Barton NC
Tuomeya sp. NH15
Clade J
Lemanea sp. SWE11
Lemanea sp. OR118
Clade H
Paralemanea sp. CA16
Paralemanea sp. CA14
Paralemanea sp. CA26
Paralemanea catenata CA24
Paralemanea catenata IDAHO
Paralemanea sp. Kenya 12
Clade I
B. atrum BI13
Batrachospermum sp. EU636741
B. heterocorticum EU636740
B. boryanum FL60
B. involutum San Marcos TX
B. boryanum ONBSC2d
Clade G (Section Batrachospermum)
B. atrum SCO14
Batrachospermum sp. BI13
Batrachospermum sp. 5 Kenya 9
Clade F (Section Setacea)
B. macrosporum EU636759
B. macrosporum MS1a
B. macrosporum MS9
Batrachospermum sp. NSCB
Batrachospermum sp. NRC
B. macrosporum EU636756
B. macrosporum EU636747
B. macrosporum EU636748
Clade E (Section Aristata)
Thorea ramosa
Thorea okoidai
Thorea sp. NY
Thorea sp. TX SM
Thorea violacea
Clade D
B. turfosum EU636745
B. turfosum MINN
B. turfosum EU095972
B. turfosum EU636746
B. turfosum Moscow B
B. turfosum JPC
Clade C (Section Turfosa)
B. procarpum LA22
B. procarpum LA27
B. breutelii
Section Gonimopropagulum
B. intortum EU636717
Batrachospermum sp. 1 Kenya 7
Batrachospermum sp. 2 Kenya 7
B. globosporum TX9
B. louisianae TX36
B. confusum ON
B. globosporum AZ10a
B. ambiguum BLZ13a
B. ambiguum CR22
B. ambiguum EU636723
B. ambiguum CR27
B. ambiguum EU095970
B. ambiguum EU636724
Clade A (Section Contorta)
B. virgatum helminthosum RI40
B. helminthosum RI1
B. virgatum CT24
B. helminthosum EU073845
B. helminthosum EU073844
B. helminthosum Oak Ridge
Batrachospermum sp. Kenya 9
B. helminthosum EU073847
B. helminthosum EU073848
Clade B (Section Virescentia)
Neodilsea sp. AY970617
Outgroup
0.1

Figure 6: Maximum Parsimony (MP) tree derived from COI sequence data.

Tree represents the one most parsimonious tree based on 334 parsimonous-informative characters of well-aligned COI sequences in the order Batrachospermales and Thoreales (tree length = 2170. consistency index (CI) = 0.3255 and retention index (RI) = 0.7842). The numbers above the tree branch represents bootstrap support of 1,000 replicates. Nodes with <50% bootstrap support are not labelled.

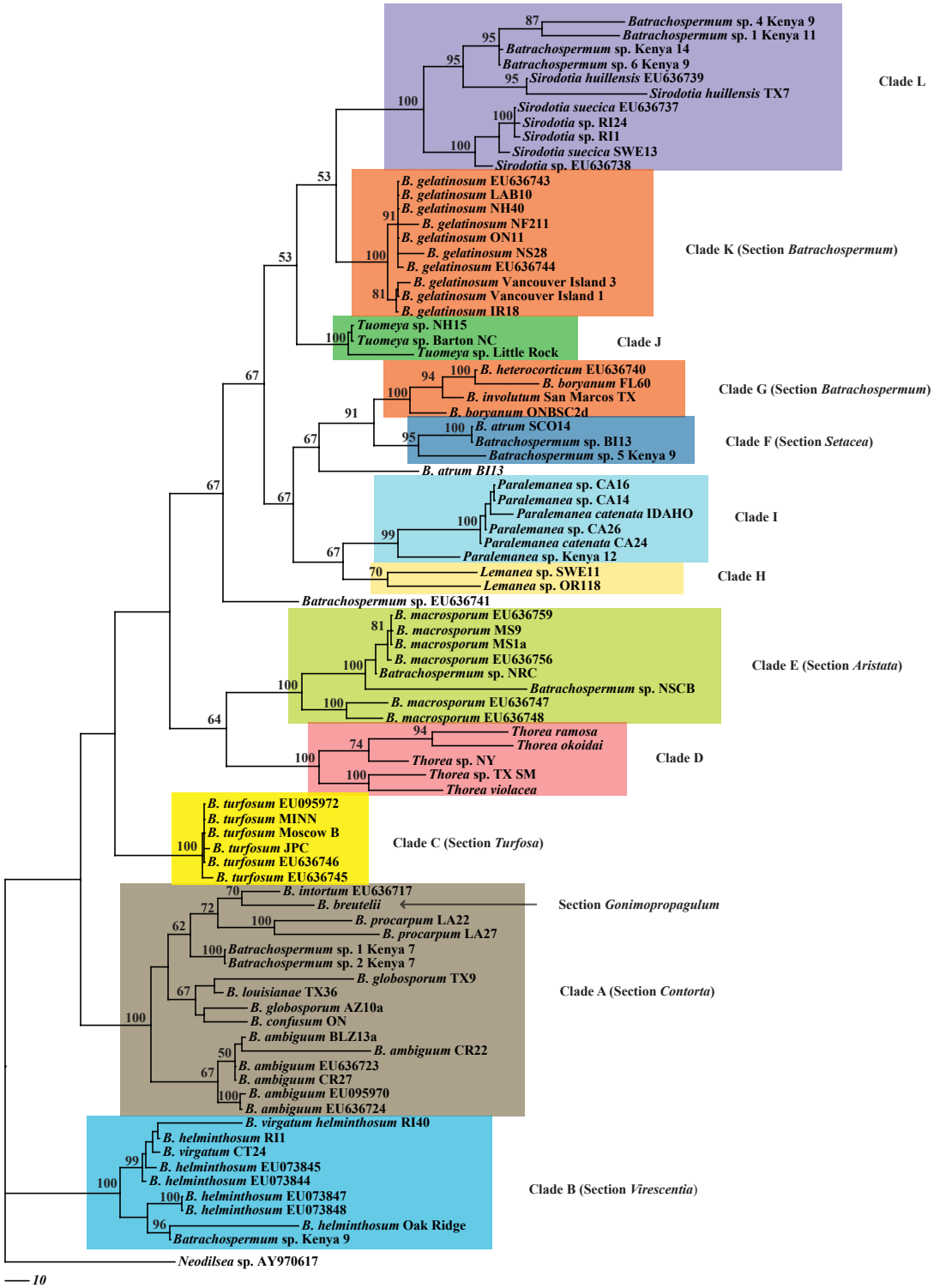
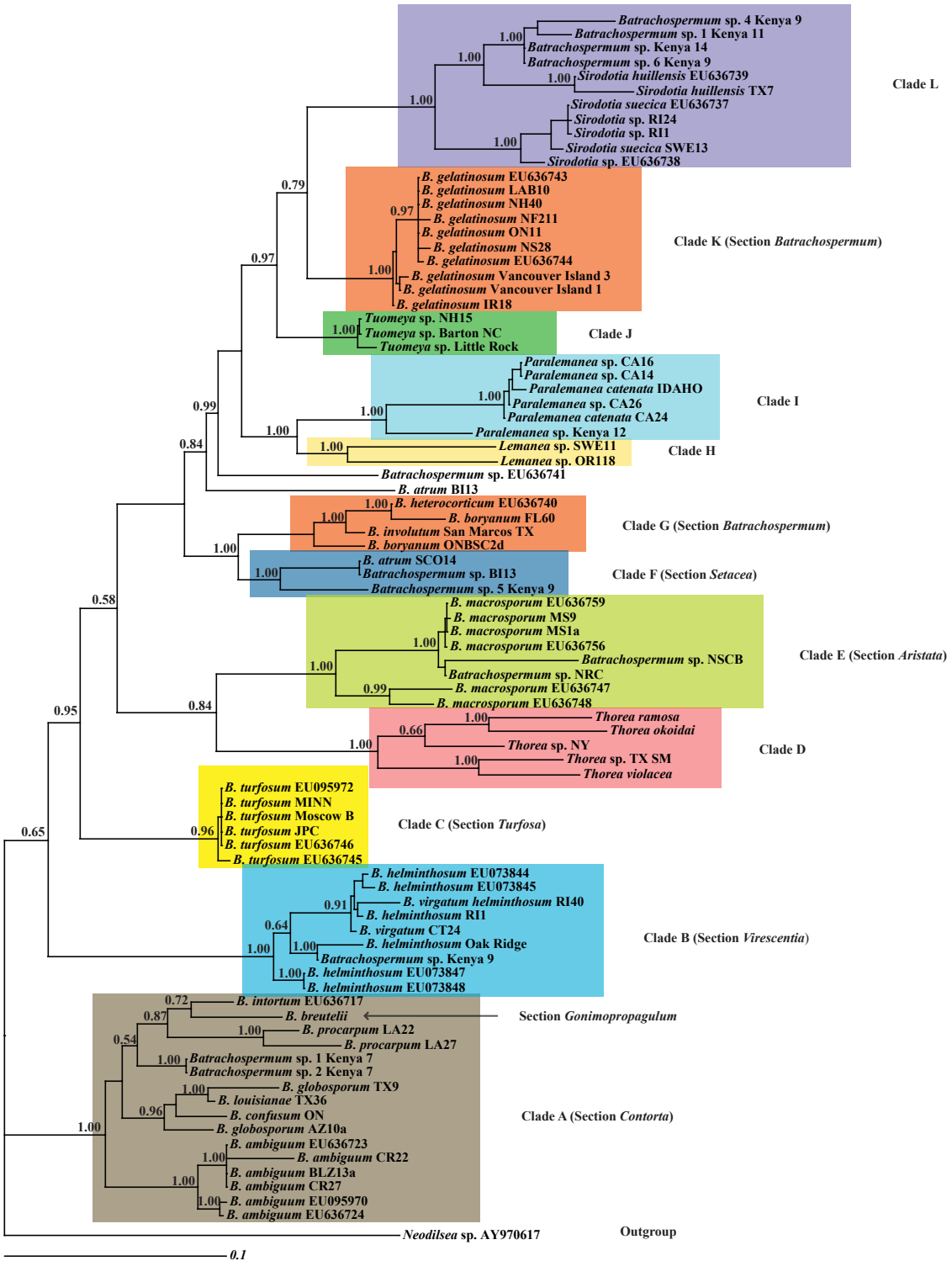

Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. 1 Kenya 11
Batrachospermum sp. Kenya 14
Batrachospermum sp. 6 Kenya 9
Sirodotia huillensis EU636739
Sirodotia huillensis TX7
Sirodotia suecica EU636737
Sirodotia sp. RI24
Sirodotia sp. RI1
Sirodotia suecica SWE13
Sirodotia sp. EU636738
Clade L
B. gelatinosum EU636743
B. gelatinosum LAB10
B. gelatinosum NH40
B. gelatinosum NF211
B. gelatinosum ON11
B. gelatinosum NS28
B. gelatinosum EU636744
B. gelatinosum Vancouver Island 3
B. gelatinosum Vancouver Island 1
B. gelatinosum IR18
Clade K (Section Batrachospermum)
Tuomeya sp. NH15
Tuomeya sp. Barton NC
Tuomeya sp. Little Rock
Clade J
B. heterocorticum EU636740
B. boryanum FL60
B. involutum San Marcos TX
B. boryanum ONBSC2d
Clade G (Section Batrachospermum)
B. atrum SCO14
Batrachospermum sp. BI13
Batrachospermum sp. 5 Kenya 9
B. atrum BI13
Clade F (Section Setacea)
Paralemanea sp. CA16
Paralemanea sp. CA14
Paralemanea catenata IDAHO
Paralemanea sp. CA26
Paralemanea catenata CA24
Paralemanea sp. Kenya 12
Clade I
Lemanea sp. SWE11
Lemanea sp. OR118
Clade H
Batrachospermum sp. EU636741
B. macrosporum EU636759
B. macrosporum MS9
B. macrosporum MS1a
B. macrosporum EU636756
Batrachospermum sp. NRC
Batrachospermum sp. NSCB
B. macrosporum EU636747
B. macrosporum EU636748
Clade E (Section Aristata)
Thorea ramosa
Thorea okoidai
Thorea sp. NY
Thorea sp. TX SM
Thorea violacea
Clade D
B. turfosum EU095972
B. turfosum MINN
B. turfosum Moscow B
B. turfosum JPC
B. turfosum EU636746
B. turfosum EU636745
Clade C (Section Turfosa)
B. intortum EU636717
B. breutelii
Section Gonimopropagulum
B. procarpum LA22
B. procarpum LA27
Batrachospermum sp. 1 Kenya 7
Batrachospermum sp. 2 Kenya 7
B. globosporum TX9
B. louisianae TX36
B. globosporum AZ10a
B. confusum ON
B. ambiguum BLZ13a
B. ambiguum CR22
B. ambiguum EU636723
B. ambiguum CR27
B. ambiguum EU095970
B. ambiguum EU636724
Clade A (Section Contorta)
B. virgatum helminthosum RI40
B. helminthosum RI1
B. virgatum CT24
B. helminthosum EU073845
B. helminthosum EU073844
B. helminthosum EU073847
B. helminthosum EU073848
B. helminthosum Oak Ridge
Batrachospermum sp. Kenya 9
Clade B (Section Virescentia)
Neodilsea sp. AY970617
10

Figure 7: Maximum Likelihood (ML) Tree from COI Sequence Data

Numbers above internal node represent consensus values from Bayesian analysis. Likelihood settings were model=GTR + G, nst=6, nucleotide frequencies (A=0.23670 C=0.19600 G=0.19010 T=0.37720), rate=gama, shape=0.2484, nrep=10, branch swapping=TBR. Nodes with <50% bootstrap support are not labelled.


Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. 1 Kenya 11
Batrachospermum sp. Kenya 14
Batrachospermum sp. 6 Kenya 9
Sirodotia huillensis EU636739
Sirodotia huillensis TX7
Sirodotia suecica EU636737
Sirodotia sp. RI24
Sirodotia sp. RI1
Sirodotia suecica SWE13
Sirodotia sp. EU636738
Clade L
B. gelatinosum EU636743
B. gelatinosum LAB10
B. gelatinosum NH40
B. gelatinosum NF211
B. gelatinosum ON11
B. gelatinosum NS28
B. gelatinosum EU636744
B. gelatinosum Vancouver Island 3
B. gelatinosum Vancouver Island 1
B. gelatinosum IR18
Clade K (Section Batrachospermum)
Tuomeya sp. NH15
Tuomeya sp. Barton NC
Tuomeya sp. Little Rock
Clade J
Paralemanea sp. CA16
Paralemanea sp. CA14
Paralemanea catenata IDAHO
Paralemanea sp. CA26
Paralemanea catenata CA24
Paralemanea sp. Kenya 12
Clade I
Lemanea sp. SWE11
Lemanea sp. OR118
Clade H
Batrachospermum sp. EU636741
B. atrum BI13
B. heterocorticum EU636740
B. boryanum FL60
B. involutum San Marcos TX
B. boryanum ONBSC2d
Clade G (Section Batrachospermum)
B. atrum SCO14
Batrachospermum sp. BI13
Batrachospermum sp. 5 Kenya 9
Clade F (Section Setacea)
B. macrosporum EU636759
B. macrosporum MS9
B. macrosporum MS1a
B. macrosporum EU636756
Batrachospermum sp. NSCB
Batrachospermum sp. NRC
B. macrosporum EU636747
B. macrosporum EU636748
Clade E (Section Aristata)
Thorea ramosa
Thorea okoidai
Thorea sp. NY
Thorea sp. TX SM
Thorea violacea
Clade D
B. turfosum EU095972
B. turfosum MINN
B. turfosum Moscow B
B. turfosum JPC
B. turfosum EU636746
B. turfosum EU636745
Clade C (Section Turfosa)
B. helminthosum EU073844
B. helminthosum EU073845
B. virgatum helminthosum RI40
B. helminthosum RI1
B. virgatum CT24
B. helminthosum Oak Ridge
Batrachospermum sp. Kenya 9
B. helminthosum EU073847
B. helminthosum EU073848
Clade B (Section Virescentia)
B. intortum EU636717
B. breutelii
Section Gonimopropagulum
B. procarpum LA22
B. procarpum LA27
Batrachospermum sp. 1 Kenya 7
Batrachospermum sp. 2 Kenya 7
B. globosporum TX9
B. louisianae TX36
B. confusum ON
B. globosporum AZ10a
B. ambiguum EU636723
B. ambiguum CR22
B. ambiguum BLZ13a
B. ambiguum CR27
B. ambiguum EU095970
B. ambiguum EU636724
Clade A (Section Contorta)
Neodilsea sp. AY970617
Outgroup
0.1

3.2 *RBC*L GENE SEQUENCE ANALYSES

The Ribulose 1, 5-bisphosphate carboxylase/oxygenase (*rbc*L) gene was successfully amplified and sequenced for 81 samples (Figure 3, Table 2) for the freshwater orders Batrachospermales and Thoreales. In addition, 26 sequences from GenBank were added and including three species of the genus *Palmaria* for the outgroup (Table 4). The gene contained no insertions or deletions, which enabled a final alignment of 991 bp to be constructed (out of 1261). A Neighbor-Joining (NJ) tree analysis of the *rbc*L gene is illustrated in Figure 8, and parsimony analysis of 388 (39.15%) parsimonious-informative characters resulted in one most parsimonious tree shown in Figure 9 with a tree length of 2032, CI=0.330, and RI=0.853. The maximum likelihood (ML) tree (Figure 10) is similar in topology to that of the ones generated by both Bayesian analyses and phyML aLRT (both not shown), hence their branch support Bayesian (above branch) and phyML aLRT (below branch) are shown on the ML tree. With the data set employed in this study, the order Batrachospermales is monophyletic showing five paraphyletic genera *Batrachospermum* (represented by sections) ,*Sirodotia* (NJ, 100% bootstrap; MP, 100%; ML, 1.00/1.00 support) belonging to the family Batrachospermaceae, *Lemanea* (NJ, 100% bootstrap; MP, 100% bootstrap; ML, 1.00/0.96 support) and *Paralemanea* (NJ, 100% bootstrap; MP, 100% bootstrap; ML, 0.93/0.53 support) belonging to the family Lemaneaceae and genus *Thorea* belonging to the family Thoreaceae. The third genus of the family Batrachospermaceae, *Tuomeya*, although represented by two samples *T. americana* (AF029159 and DQ523253) do not group together. Instead *T. americana* AF029159 is shown to clade within section *Batrachospermum* in NJ (Clade I, Figure 8) with 95% bootstrap support and as a sister taxon with *B. gelatinosum* in MP (Clade I, Figures 9) with 100% bootstrap and in ML (Clade I, Figure 10) with 1.00/0.90 support. *T. americana* DQ449029 on the other hand is shown to cluster within genus *Sirodotia* and well-supported (NJ, 100% bootstrap; MP, 100% bootstrap support; ML, 1.00/0.90 support). Thus, genus *Tuomeya* is paraphyletic. Also with such strong

clustering support for both *T. americana* (AF029159 and DQ523253), it could be that both taxa were morphologically misidentified or mislabeled in GenBank.

Genus *Batrachospermum*, the largest of the four Batrachospermaceae genera is represented by seven monophyletic well-supported sections *Contorta* (NJ, 100% BS; MP, 100% BS; ML, 1.00/0.95 support), *Aristata* (NJ, 100% BS; MP, 100% BS; ML, 1.00/1.00 support), *Setacea* (NJ, 100% BS; MP, 100% BS; ML, 1.00/1.00 support), *Batrachospermum* (NJ, 83% BS and 95 BS (Clades H and I, Figure 8); MP, 77 BS and 91 BS (Clades H and I, Figure 9); ML, 1.00/0.99 and 1.00/0.90 support), *Gonimopropagulum*, *Virescentia* (NJ, 100% BS; MP, 100% BS; ML, 1.00/1.00 support), *Turfosa* (NJ, 100% BS; MP, 100% BS; ML, 1.00/0.99 support) (Clades: E, C, B, (H and I), F, D and B respectively). One apparent feature of these trees (Figures 8 and 9) is that members of the Thoreaceae form a well-supported clade (NJ, 100% BS; MP, 100% BS). The relationship of Thoreales as a separate order from the order Batrachospermales is well-supported (NJ, 100% BS; MP, 100%; ML, 1.00/1.00 support) and clearly illustrated by all three phylogenetic methods (Figures 8, 9 and 10). The order Batrachospermales forms a moderately supported entity (NJ, 81% BS; MP, 82% BS; ML, 1.00/1.00 support). Within the Thoreaceae all members are closely associated (NJ, 100%; MP, 100%; ML, 1.00/1.00 support) and in addition all three representatives of *T. hispida* (AB159652, AB159653, AF506270) and the one sample of *T. ramosa* form a solid cluster (Clade A) that is well-supported by bootstrap analysis (NJ, 100 BS; MP; 100% BS; ML, 1.00/1.00 support) (Figure 8,9 and 10). Interestingly, *T. ramosa* is closely associated with all three *T. hispida* (AB159652, AB159653, AF506270) in the NJ (100% BS), in MP (100% BS) and in ML (1.00/1.00 support). In addition, *T. violacea* is closely positioned with this cluster (NJ, 100% BS; MP, 100% BS; ML, 1.00/1.00 support). The other two remaining collections of *T. violacea* (AB159657 and AF506271) are well supported as being distinct from *T. violacea* (AF506269), *T. hispida*, *T. ramosa* and *Thorea* sp. (NY); hence *T. violacea* is paraphyletic (Figures 8,9 and 10).

Figure 8: Neighbor-Joining tree derived from *rbc*L sequence data.

NJ tree from analysis of 991 nucleotides of the *rbc*L gene. Numbers above branches represents bootstrap confidence values for the grouping as a percentage of 1000 replicates. Members of the genus *Palmaria* are used as outgroup.

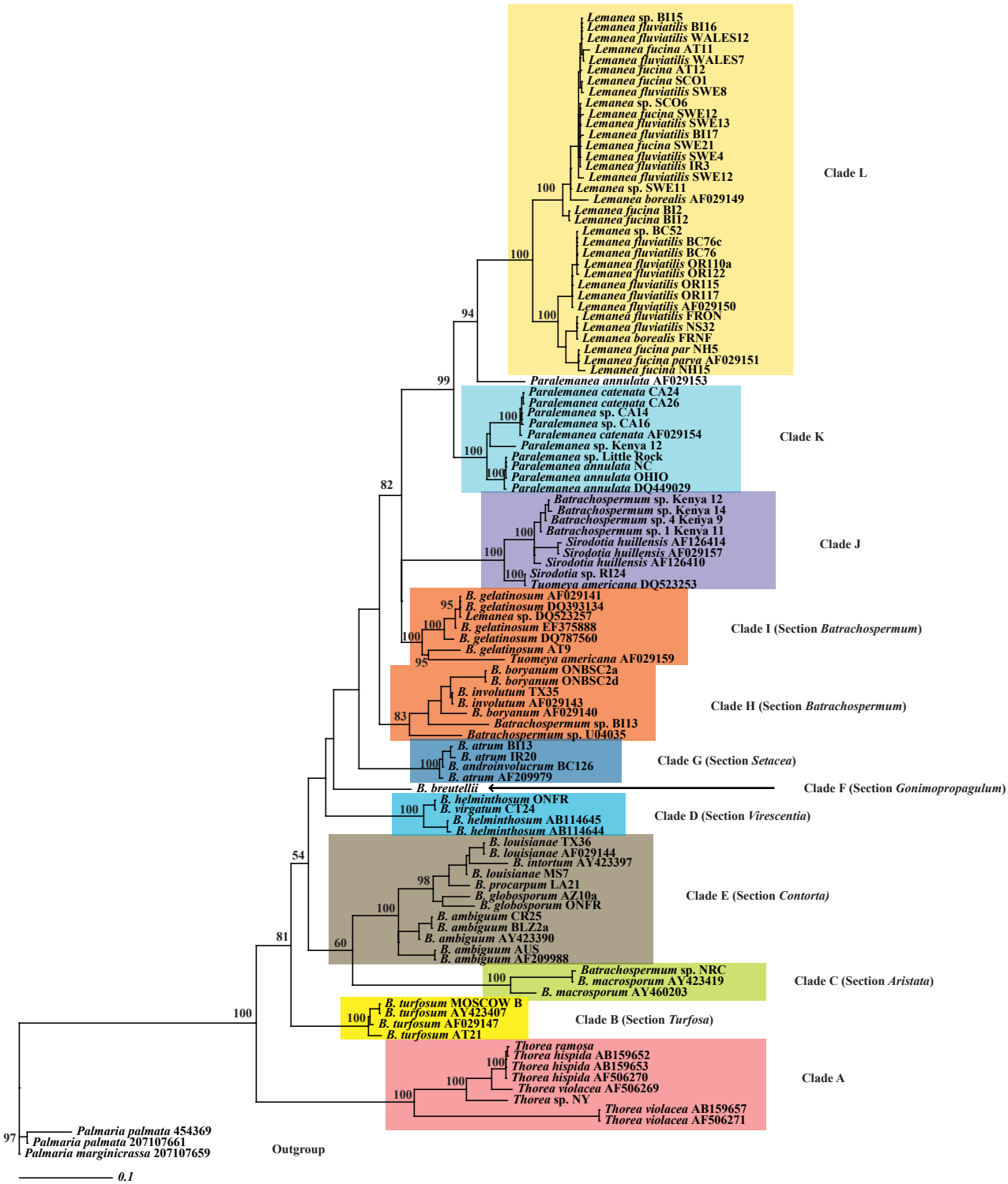
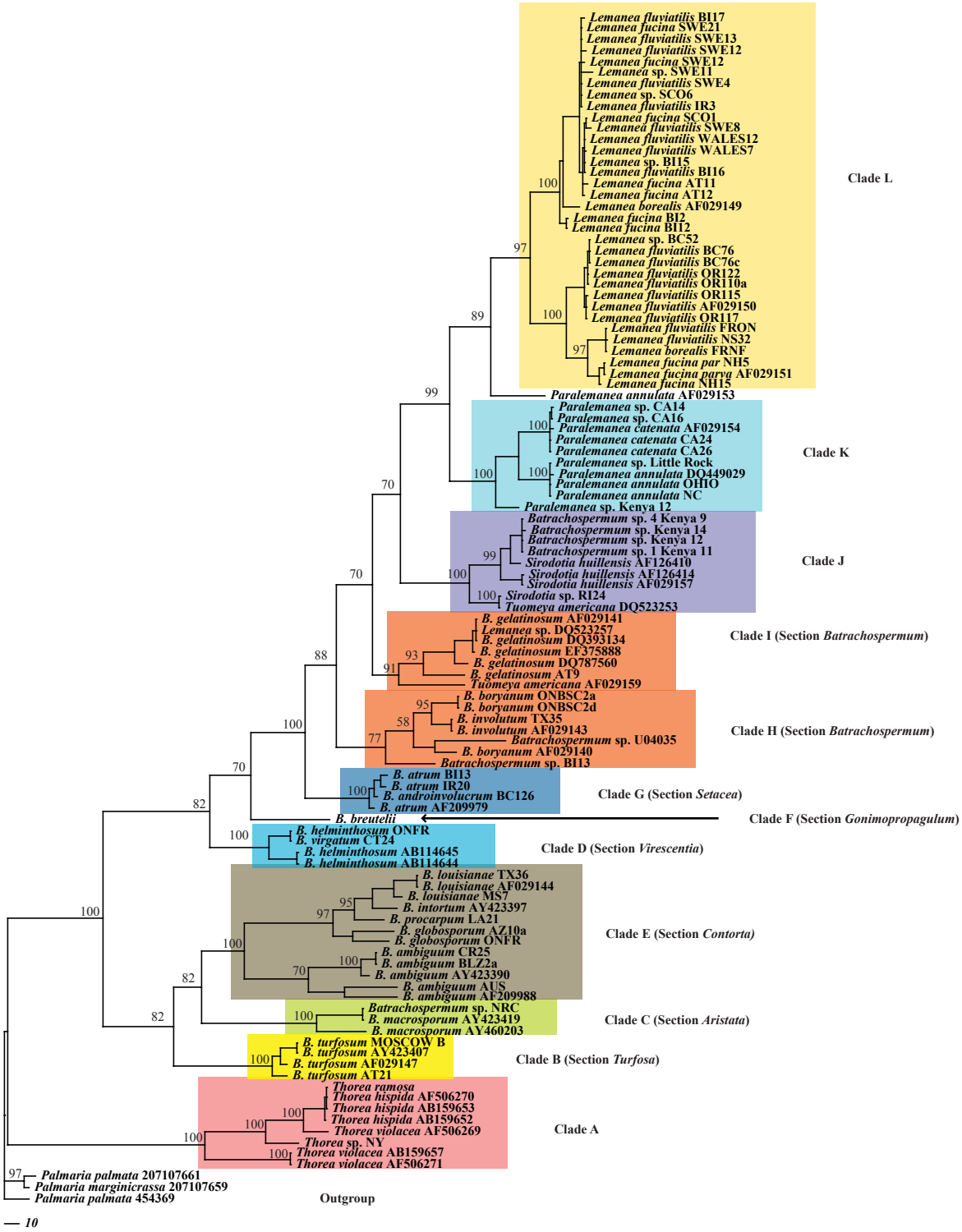

Lemanea sp. BI15
Lemanea fluviatilis BI16
Lemanea fluviatilis WALES12
Lemanea fucina AT11
Lemanea fluviatilis WALES7
Lemanea fucina AT12
Lemanea fucina SCO1
Lemanea fluviatilis SWE8
Lemanea sp. SCO6
Lemanea fucina SWE12
Lemanea fluviatilis SWE13
Lemanea fluviatilis BI17
Lemanea fucina SWE21
Lemanea fluviatilis SWE4
Lemanea fluviatilis IR3
Lemanea fluviatilis SWE12
Lemanea sp. SWE11
Lemanea borealis AF029149
Lemanea fucina BI2
Lemanea fucina BI12
Lemanea sp. BC52
Lemanea fluviatilis BC76c
Lemanea fluviatilis BC76
Lemanea fluviatilis OR110a
Lemanea fluviatilis OR122
Lemanea fluviatilis OR115
Lemanea fluviatilis OR117
Lemanea fluviatilis AF029150
Lemanea fluviatilis FRON
Lemanea fluviatilis NS32
Lemanea borealis FRNF
Lemanea fucina par NH5
Lemanea fucina parva AF029151
Lemanea fucina NH15
Clade L
Paralemanea annulata AF029153
Paralemanea catenata CA24
Paralemanea catenata CA26
Paralemanea sp. CA14
Paralemanea sp. CA16
Paralemanea catenata AF029154
Paralemanea sp. Kenya 12
Paralemanea sp. Little Rock
Paralemanea annulata NC
Paralemanea annulata OHIO
Paralemanea annulata DQ449029
Clade K
Batrachospermum sp. Kenya 12
Batrachospermum sp. Kenya 14
Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. 1 Kenya 11
Sirodotia huillensis AF126414
Sirodotia huillensis AF029157
Sirodotia huillensis AF126410
Sirodotia sp. RI24
Tuomeya americana DQ523253
Clade J
B. gelatinosum AF029141
B. gelatinosum DQ393134
Lemanea sp. DQ523257
B. gelatinosum EF375888
B. gelatinosum DQ787560
B. gelatinosum AT9
Tuomeya americana AF029159
Clade I (Section Batrachospermum)
B. boryanum ONBSC2a
B. boryanum ONBSC2d
B. involutum TX35
B. involutum AF029143
B. boryanum AF029140
Batrachospermum sp. BI13
Batrachospermum sp. U04035
Clade H (Section Batrachospermum)
B. atrum BI13
B. atrum IR20
B. androinvolucrum BC126
B. atrum AF209979
Clade G (Section Setacea)
B. breutellii
Clade F (Section Gonimopropagulum)
B. helminthosum ONFR
B. virgatum CT24
B. helminthosum AB114645
B. helminthosum AB114644
Clade D (Section Virescentia)
B. louisianae TX36
B. louisianae AF029144
B. intortum AY423397
B. louisianae MS7
B. procarpum LA21
B. globosporum AZ10a
B. globosporum ONFR
B. ambiguum CR25
B. ambiguum BLZ2a
B. ambiguum AY423390
B. ambiguum AUS
B. ambiguum AF209988
Clade E (Section Contorta)
Batrachospermum sp. NRC
B. macrosporum AY423419
B. macrosporum AY460203
Clade C (Section Aristata)
B. turfosum MOSCOW B
B. turfosum AY423407
B. turfosum AF029147
B. turfosum AT21
Clade B (Section Turfosa)
Thorea ramosa
Thorea hispida AB159652
Thorea hispida AB159653
Thorea hispida AF506270
Thorea violacea AF506269
Thorea sp. NY
Thorea violacea AB159657
Thorea violacea AF506271
Clade A
Palmaria palmata 454369
Palmaria palmata 207107661
Palmaria marginicrassa 207107659
Outgroup
0.1

Figure 9: Maximum Parsimony (MP) tree derived from *rbc*L data.

Strict most-parsimonious tree from analysis of the *rbc*L gene sequence data containing 388 parsimonous informative characters. Numbers above branches represent bootstrap values from MP analysis as a percentage of 1000 replicates. Bootstrap support values of >50% for nodes shared by bootstrap consensus trees are shown. Members of the genus *Palmaria* are used as outgroup.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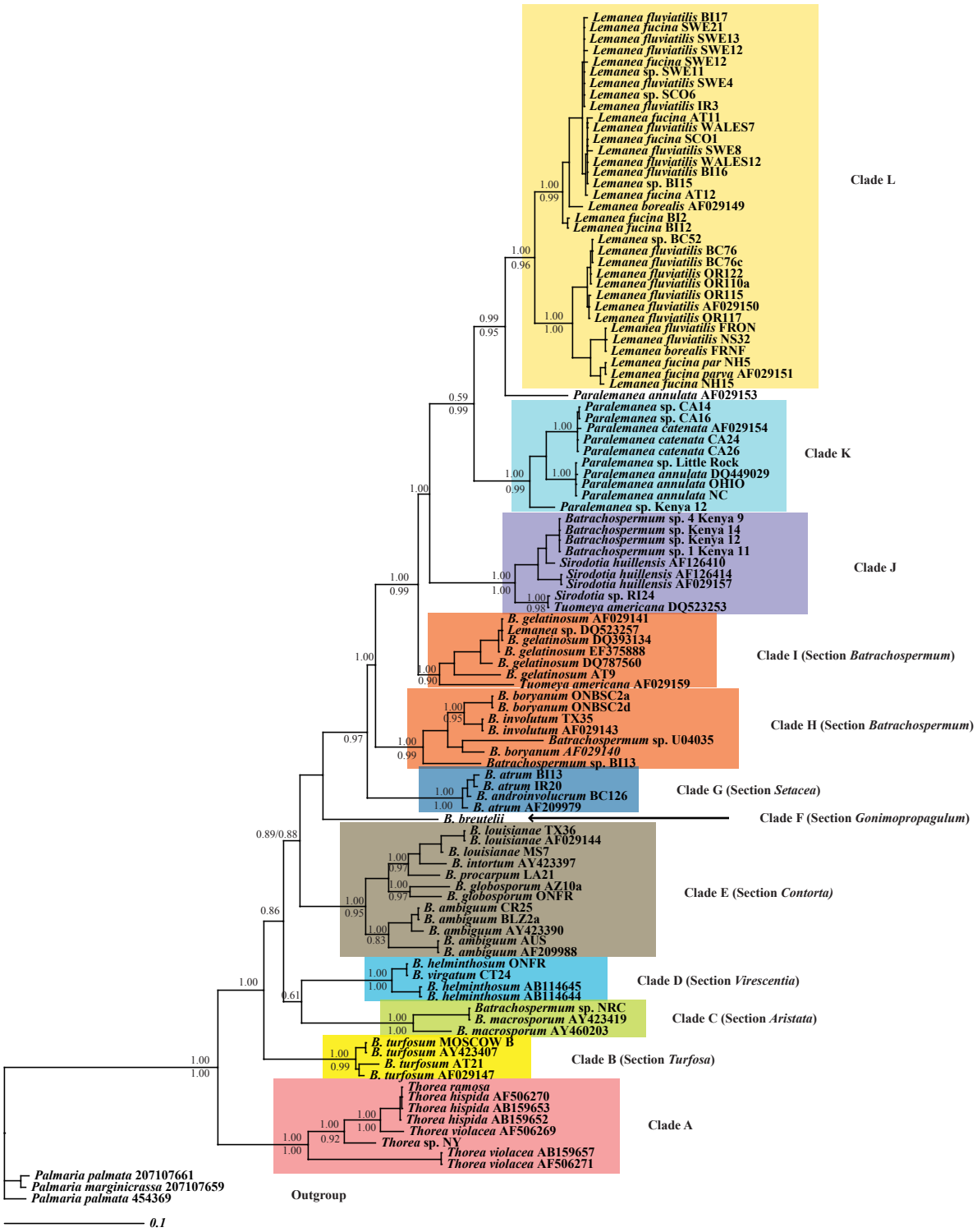
Lemanea fucina SWE21
Lemanea fluviatilis SWE13
Lemanea fluviatilis SWE12
Lemanea fucina SWE12
Lemanea sp. SWE11
Lemanea fluviatilis SWE4
Lemanea sp. SCO6
Lemanea fluviatilis IR3
Lemanea fucina SCO1
Lemanea fluviatilis SWE8
Lemanea fluviatilis WALES12
Lemanea fluviatilis WALES7
Lemanea sp. BI15
Lemanea fluviatilis BI16
Lemanea fucina AT11
Lemanea fucina AT12
Lemanea borealis AF029149
Lemanea fucina BI2
Lemanea fucina BI12
Lemanea sp. BC52
Lemanea fluviatilis BC76
Lemanea fluviatilis BC76c
Lemanea fluviatilis OR122
Lemanea fluviatilis OR110a
Lemanea fluviatilis OR115
Lemanea fluviatilis AF029150
Lemanea fluviatilis OR117
Lemanea fluviatilis FRON
Lemanea fluviatilis NS32
Lemanea borealis FRNF
Lemanea fucina par NH5
Lemanea fucina parva AF029151
Lemanea fucina NH15
Clade L
Paralemanea annulata AF029153
Paralemanea sp. CA14
Paralemanea sp. CA16
Paralemanea catenata AF029154
Paralemanea catenata CA24
Paralemanea catenata CA26
Paralemanea sp. Little Rock
Paralemanea annulata DQ449029
Paralemanea annulata OHIO
Paralemanea annulata NC
Paralemanea sp. Kenya 12
Clade K
Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. Kenya 14
Batrachospermum sp. Kenya 12
Batrachospermum sp. 1 Kenya 11
Sirodotia huillensis AF126410
Sirodotia huillensis AF126414
Sirodotia huillensis AF029157
Sirodotia sp. RI24
Tuomeya americana DQ523253
Clade J
B. gelatinosum AF029141
Lemanea sp. DQ523257
B. gelatinosum DQ393134
B. gelatinosum EF375888
B. gelatinosum DQ787560
B. gelatinosum AT9
Tuomeya americana AF029159
Clade I (Section Batrachospermum)
B. boryanum ONBSC2a
B. boryanum ONBSC2d
B. involutum TX35
B. involutum AF029143
Batrachospermum sp. U04035
B. boryanum AF029140
Batrachospermum sp. BI13
Clade H (Section Batrachospermum)
B. atrum BI13
B. atrum IR20
B. androinvolucrum BC126
B. atrum AF209979
Clade G (Section Setacea)
B. breutelii
Clade F (Section Gonimopropagulum)
B. helminthosum ONFR
B. virgatum CT24
B. helminthosum AB114645
B. helminthosum AB114644
Clade D (Section Virescentia)
B. louisianae TX36
B. louisianae AF029144
B. louisianae MS7
B. intortum AY423397
B. procarpum LA21
B. globosporum AZ10a
B. globosporum ONFR
B. ambiguum CR25
B. ambiguum BLZ2a
B. ambiguum AY423390
B. ambiguum AUS
B. ambiguum AF209988
Clade E (Section Contorta)
Batrachospermum sp. NRC
B. macrosporum AY423419
B. macrosporum AY460203
Clade C (Section Aristata)
B. turfosum MOSCOW B
B. turfosum AY423407
B. turfosum AF029147
B. turfosum AT21
Clade B (Section Turfosa)
Thorea ramosa
Thorea hispida AF506270
Thorea hispida AB159653
Thorea hispida AB159652
Thorea violacea AF506269
Thorea sp. NY
Thorea violacea AB159657
Thorea violacea AF506271
Clade A
Palmaria palmata 207107661
Palmaria marginicrassa 207107659
Palmaria palmata 454369
Outgroup
10

Figure 10: Maximum Likelihood (ML) tree infered from *rbcL* gene sequence data.

Tree highlights the relationship between the order Batrachospermales and Thoreales. Probaility values shown on tree branches represent ones from Bayesian analyses (above branch) and from PHYML aLRT (below branch).


Lemanea fluviatilis BI17
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Lemanea fluviatilis SWE12
Lemanea fucina SWE12
Lemanea sp. SWE11
Lemanea fluviatilis SWE4
Lemanea sp. SCO6
Lemanea fluviatilis IR3
Lemanea fucina AT11
Lemanea fluviatilis WALES7
Lemanea fucina SCO1
Lemanea fluviatilis SWE8
Lemanea fluviatilis WALES12
Lemanea fluviatilis BI16
Lemanea sp. BI15
Lemanea fucina AT12
Lemanea borealis AF029149
Lemanea fucina BI2
Lemanea fucina BI12
Lemanea sp. BC52
Lemanea fluviatilis BC76
Lemanea fluviatilis BC76c
Lemanea fluviatilis OR122
Lemanea fluviatilis OR110a
Lemanea fluviatilis OR115
Lemanea fluviatilis AF029150
Lemanea fluviatilis OR117
Lemanea fluviatilis FRON
Lemanea fluviatilis NS32
Lemanea borealis FRNF
Lemanea fucina par NH5
Lemanea fucina parva AF029151
Lemanea fucina NH115
Clade L
Paralemanea annulata AF029153
Paralemanea sp. CA14
Paralemanea sp. CA16
Paralemanea catenata AF029154
Paralemanea catenata CA24
Paralemanea catenata CA26
Paralemanea sp. Little Rock
Paralemanea annulata DQ449029
Paralemanea annulata OHIO
Paralemanea annulata NC
Paralemanea sp. Kenya 12
Clade K
Batrachospermum sp. 4 Kenya 9
Batrachospermum sp. Kenya 14
Batrachospermum sp. Kenya 12
Batrachospermum sp. 1 Kenya 11
Sirodotia huillensis AF126410
Sirodotia huillensis AF126414
Sirodotia huillensis AF029157
Sirodotia sp. RI24
Tuomeya americana DQ523253
Clade J
B. gelatinosum AF029141
Lemanea sp. DQ523257
B. gelatinosum DQ393134
B. gelatinosum EF375888
B. gelatinosum DQ787560
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Clade H (Section Batrachospermum)
B. atrum BI13
B. atrum IR20
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B. atrum AF209979
Clade G (Section Setacea)
B. breutelii
Clade F (Section Gonimopropagulum)
B. louisianae TX36
B. louisianae AF029144
B. louisianae MS7
B. intortum AY423397
B. procarpum LA21
B. globosporum AZ10a
B. globosporum ONFR
B. ambiguum CR25
B. ambiguum BLZ2a
B. ambiguum AY423390
B. ambiguum AUS
B. ambiguum AF209988
Clade E (Section Contorta)
B. helminthosum ONFR
B. virgatum CT24
B. helminthosum AB114645
B. helminthosum AB114644
Clade D (Section Virescentia)
Batrachospermum sp. NRC
B. macrosporum AY423419
B. macrosporum AY460203
Clade C (Section Aristata)
B. turfosum MOSCOW B
B. turfosum AY423407
B. turfosum AT21
B. turfosum AF029147
Clade B (Section Turfosa)
Thorea ramosa
Thorea hispida AF506270
Thorea hispida AB159653
Thorea hispida AB159652
Thorea violacea AF506269
Thorea sp. NY
Thorea violacea AB159657
Thorea violacea AF506271
Clade A
Palmaria palmata 207107661
Palmaria marginicrassa 207107659
Palmaria palmata 454369
Outgroup
0.1

4.0 DISCUSSION

The red algae (Rhodophyta) are a distinct lineage characterized by the lack of flagella and centrioles. In traditional taxonomic schemes, this division contains the classes Bangiophyceae and Florideophyceae. The monophyletic Florideophycidae is estimated to contain over 32 orders (Schneider and Wynne, 2007), of which the Batrachospermales and Thoreales are the focus of the present study. Results from the present study suggest it is possible to differentiate between and among red algal species employing data sequence of COI. This conclusion is similar to findings by Saunders (2005), Robba *et al.* (2006) and House *et al.* (2008). The COI gene, to a considerable extent, echoes the results of the *rbc*L gene at the terminal branches although differ at deep branching in this study.. Overall, the COI and *rbc*L gene sequence analyses suggest and raise major issues with the taxonomy of the Batrachospermales that warrant further investigation. A clearly polyphyletic grouping is indicated for members of the Batrachospermales.

4.1 GENERIC-LEVEL TAXONOMY

The order Thoreales is represented in this study by species of *Thorea* (Clade D, Figures 5, 6, and 7; Clade A, Figures 8, 9, and 10) and in all trees generated from *rbc*L gene sequence data, genus *Thorea* was not closely related to other taxa of the Batrachospermales. Consequently, the family Thoreaceae does not appear to be a natural grouping within the Batrachospermales, which is consistent with finding by Müller *et al.* (2002), who examined pit-plug structures and analyzed sequences of *rbc*L and small subunit of rRNA (18S rRNA). The previous placement of the Thoreaceae in the Batrachospermales was based in part on pit plug ultrastructure, consisting of two cap layers, the outer one of which is domed (Pueschel 1989). However, Schnepf (1992) observed that pit plugs of *T. hispida* (Thore) Desvaux (as *T. ramosissima* Bory) were variable, including ones with a plate-like outer cap layer. The latter type of plug is found in the Nemaliales, Acrochaetiales, and Palmariales (Pueschel and Cole 1982). Although the

Thoreaceae superficially resemble members of the Nemaliales in being multiaxial, the inclusion of the Thoreaceae in the Nemaliales, Acrochaetiales, or Palmariales has been observed to lack support (e.g. Vis *et al.*, 1998). The *rbc*L sequence data in the present study is in accordance with an earlier phylogenetic analysis of the Batrachospermalean genera based on a variety of morphological and cellular characteristics by Entwisle and Necchi (1992). Entwisle and Necchi (1992) concluded that the Thoreaceae may have evolved separately from the genera Batrachospermaceae and Lemaneaceae. Some of their cladograms also placed *Thorea* on an early branch, indicating that multiaxial thallus construction may be a plesiomorphic character. One conclusion that could have been drawn, especially from the 18S gene data was that the Thoreaceae be elevated to the status of order. The analysis conducted by Müller *et al.* (2002) on other specimens of *Thorea* and *Nemalionopsis,* did in fact elevate the Thoreaceae to the order status. Aimed at resolving the taxonomic and phylogenetic status of the Thoreaceae, Müller *et al.* (2002) examined specimens of *Nemalionopsis* and *Thorea*, using as already mentioned above, their pit-plug ultrastructure and sequence data information from their *rbc*L and small subunit of rRNA (18S rRNA) genes. Phylogenetic trees derived from the two genes by Müller and colleagues showed the Thoreaceae to be a well-supported monophyletic clade that groups independently from the Batrachospermaceae and Lemaneaceae, the other two families of the order Batrachospermales. This seems also to be supported by Müller *et al.* (2002) evaluation of the pit plugs of the gametophyte and chantransia stages of the Thoreaceae. The gamotophyte and chantransia phases revealed the Thoreaceae to contain two cap layers, with the outer one usually having plate-like shape. In addition, no pit plug cap membrane was observed, suggesting also that Thoreaceae has been misclassified in the Batrachospermales. Thus, the elevation of the Thoreaceae by the authors to its own order, Thoreales and is supported in the here. Unlike the pit-plug ultrastructure, *rbc*L (Müller *et al.*,2002; present study), and 18S rRNA genes sequence data analyses, in the COI sequence analyses, the genus *Thorea* (Clade D, Figures 5, 6, and 7) also well-supported, is observed to group within the order Batrachospermales, forming a sister taxa with section *Aristata* (Clade E, Figures 5, 6, and 7). This observation raises the question whether Thorea in fact belong to the status of order. Since by account of the COI gene it forms a sister taxon with a section, should the sections be

elevated to the status of genera, then perhaps Thoreales should be brought down to the same taxonomic level. Nevertheless, although COI gene sequence data for Thoreales is incongruent with the pit-plug ultrastructure, *rbc*L (Müller *et al.*, 2002; present study), and 18S rRNA genes sequence data analyses, it is not unexpected since DNA barcoding does not aim at inferring species relationships but rather identifying and grouping species. Exploration of any biogeographic trends within the data set for both COI and *rbc*L genes in the present study revealed very few biogeographic trends, examples of which include North American and European separation of *Lemanea* sp. in the r*bc*L gene sequence. More samples from distant geographic locations would be required to establish any biogeographic patterns.

At the generic level, genus the *Sirodotia* is well-supported in the analyses of both COI and *rbc*L genes (Clade F, Figures 5, 6 and 7; Clade J, Figures 8, 9, and 10). In both the COI and *rbc*L sequence data analyses, *Sirodotia huillensis* is observed to group closely with samples identified as *Batrachospermum* sp. from Kenya. This grouping is expected since occurrence of *Sirodotia* is been reported in Africa (Rantzien, 1950) and *Batrachospermum huillense* Welwitsch ex W. et G. S. West (1897) from Angola, Africa was assigned to the genus *Sirodotia* as *Sirodotia huillensis* by Skuja (1931). Nonetheless, the African continent has not been well studied for members of the freshwater red algae and this is likely due to the lack of collection and research. The genus *Sirodotia* is viewed to be closely related to the genus *Batrachospermum* (Vis and Sheath, 1999). Considerable debate as to whether the morphological characters used to differentiate this section were sufficient to remain a genus or rather be placed as a section under the genus *Batrachospermum* has been ongoing (Necchi *et al.*, 1992). To clarify the taxonomic status of *Sirodotia* from *Batrachospermum*, a molecular study by Vis *et al.* (1998) using 18S rDNA and *rbc*L genes sequence data revealed a clear distinct clade of *Sirodotia* from a paraphyletic *Batrachospermum*; however, the taxonomic ranking of *Sirodotia* was unresolved. There was a consensus by authors that the combined morphometric features and sequence analysis define a distinct group whether it be ranked taxonomically as a genus or as a section of *Batrachospermum* (Necchi and Entwisle, 1990; Necchi *et al.*, 1993; Vis *et al.*, 1998). Previous studies of members of genus *Sirodotia* in North

America using multivariate morphometrics and image analysis by Orlando Jr. *et al.* (1993) revealed well-delineated species (*S. huillensis, S. suecica,* and *S. tenuissima*) differentiated on the basis of whorl shape and degree of separation at maturity. Molecular studies to determine phylogenetic relationship of this genus by Vis and Sheath (1999) using both RUBISCO large and small (*rbc*L, *rbc*S) genes revealed *S. suecica* and *S. tenuissima* to be paraphyletic and *S. huillensis* to be monophyletic by both the *rbc*L and the *rbc*L-S spacer and partial *rbc*S genes sequence data. The ITS1-5.8S rDNA-ITS2 region revealed little sequence divergence (~2%) between *S. suecica* and *S. tenuissima*; hence, Vis and Sheath (1999) suggested synonymizing the two species, with *S. suecica* taking priority and continually recognize *S. huillensis* as a distinct clade. In North America only *S. huillensis* and *S. suecica* have been reported and are observed to scatter in their occurrence but appear to have phylogeographic trends similar to those reported in other parts of the world. Vis and Sheath (1999) analyses of *S. huillensis* suggest possible geographic patterns in this taxon. Samples from the same geographic region, east Texas and east Mexico, appeared to be closely related based on *rbc*L and the *rbc*L-S spacer and partial *rbc*S genes sequence data. The collections from Costa Rica, a relatively distant location from Texas and Mexico, were observed to significantly differ in *rbc*L sequence variation (2.6%). This level of divergence exceeded previously reported variation of geographically distant collections of taxa belonging to the Batrachospermales (Vis *et al.*, 1998). The difference in sequence divergence between the two geographic locations the authors postulated could be due to much longer divergence time of the Costa Rica samples from the Texas and Mexico collections. In the present study however, no particular biogeographic trends are observed for members of the genus *Sirodotia* in both COI and *rbc*L gene sequence data analyses (Clade L, Figures 5, 6, and 7; Clade J, Figures 8, 9, and 10). In both the COI and *rbc*L gene sequence data analyses, the genus *Sirodotia* is observed to include haplotypes from Kenya identified in the field as *Batrachospermum* species. These appear as *Sirodotia* species based on these molecular analyses, although proper identification is yet to be carried out. These specimens from Kenya are observed to group distinct from the other *Sirodotia* species probably due to all specimens being localized and genetically identical. In the present study, the COI gene sequence data places the *Sirodotia* as a sister taxon with section

Batrachospermum Clade E (Figures 5, 6, 7) both of which form a sister taxon with genus *Tuomeya*. Notebably, a genus forming sister taxon with a section raises questions as to the validity of these taxonomic rankings. The *rbc*L gene sequence data analyses in the present study places the genus *Sirodotia* as a sister taxon with family Lemaneaceae, both of which form a sister taxon with the cryptic section *Batrachospermum* again questioning the validity of these taxonomic rankings. Clearly the both the COI and *rbc*L genes sequence data indicate. No biogeographic trend can be established for this genus in the present study. More specimens from North America, Europe and Africa would be needed to do establish any trends.

The genus *Tuomeya* was initially differentiated from other members of *Batrachospermum* by having densely branched, cartilaginous, and pseudoparenchymatous thallus with lateral whorls that are compacted and radiate from a uniseriate axis (Harvey, 1858). Following Harvey (1858), Setchell (1890) documented plants that appeared in-between *Batrachospermum* and *Lemanea*. Proposals of *Tuomeya* to be reduced to a section of genus *Batrachospermum* was put forth by Necchi and Entwisle (1990), but a study by Kaczmarczyk *et al.* (1992) verified the previous classification of *Tuomeya* as an independent genus and recommended retaining *Tuomeya* at the genus level. Using multivariate morphometric, Kaczmarczyk *et al.* (1992) observed *Tuomeya* to differ from *Batrachospermum* in its pseudoparenchymatous growth as well as its carpogonia by having trichogynes that are oblique to perpendicular. Furthermore, *Tuomeya* is observed to be unique in having a gametophyte that develops from a mass of undifferentiated basal cells (Feng *et al.*, 2007). This recommendation by Kaczmarczyk *et al.* (1992) was supported by Feng *et al.* (2007) in their study of *Tuomeya*, newly reported from China. Feng *et al.* (2007) examination of *Tuomeya* specimens from China using the *rbc*L gene sequence data and BLAST search resulted in close similarity of the China specimens with ones from North America (GenBank accession number AF029159) with 85.57% identity. Feng *et al.* (2007) noticed sequence variation of 14.51% between the Chinese population and North Carolina specimen (DQ523253) and 13.18% sequence divergence between the China populations and the sample (AF029352). Nonetheless,

sequence divergences of only 0.55% - 10.37% have been reported for North America in previously published data. As a consequence, the authors hypothesized the discrepancy in sequence divergence between the China and North American samples of *Tuomeya* could be accredited to biogeographic separation. *Tuomeya americana* has been referred to as unique to North America (Sheath, 2003) although it been reported to occur in South Africa (Borge, 1928), Finland (Eloranta and Kwandrans, 1996), India (Babu and Baluswami, 2005), and China (Feng *et al.*, 2007). Like the genus *Sirodotia*, *Tuomeya* in the COI gene sequence analyses is well-supported in the present study; however, in the *rbc*L gene sequence analyses (Figures 8, 9 and 10), the two *Tuomeya* species do not form a coherent grouping. Instead, *Tuomeya americana* AF029159, that was observed to be highly similar to the China *Tuomeya* population reported by Feng *et al.* (2007), is observed to form a sister taxa with the subclade containing *B. gelatinosum* (Clade D, Figures 8, 9 and 10). Additionally, *Tuomeya americana* DQ523253 is shown to form a sister taxon with *Sirodotia* sp. RI24 (Clade J, Figures 8, 9, and 10). The misplacement of these two *Tuomeya* species in the phylogenies could be attributed to either morphological misidentification by the authors or mislabeling of the taxa before submission to GenBank. It could also suggest an unnamed clade of *Tuomeya* with poor taxa representation, although the former hypothesis is much more plausible. Pairwise distance analysis reveal sequence variation of 7.9% between *B. gelatinosum* AT9 and *T. americana* (AF029159) and sequence divergence of 10.9% between *T. americana* (AF029159) and *T. americana* (DQ523253). The difference in sequence divergence between *B. gelatinosum* AT9 and *T. americana* (AF029159) versus *T. americana* (AF029159) and *T. americana* (DQ523253), although small suggest *T. americana* (AF029159) is closely related to *B. gelatinosum* AT9 than it is to *T. americana* (DQ523253). Thus suggesting both *T. americana* (AF029159) and *T. americana* (DQ523253) have been mislabeled or misidentified. The genus *Tuomeya* is evidently paraphyletic since it does not contain the two groups, genus *Sirodotia* and section *Batrachospermum*. Again the taxonomic status of the genus is brought into question. Comparison cannot be made with the *rbc*L gene sequence since no credible taxa of *Tuomeya* are represented in the data set as noted above.

The family Lemaneaceae is represented in this study by both genera *Lemanea* and *Paralemanea*. In the COI sequence analyses, genus *Lemanea* is represented by two samples that form a sister taxon with each other and moderately supported (Clade H, Figures 5, 6, and 7). In the *rbc*L sequence analyses however, *Lemanea* is made up of numerous species (*L. fucina, L. fluviatilis* and *L. borealis*) and forms a well-supported clade (Clade L, Figures 8, 9, and 10). In the *rbc*L data sequence analyses, clear biogeographic trends of European specimen from North American specimens is observed for the genus *Lemanea* Clade L (Figures 8, 9, and 10). Specimens from Wales, British Isles, Scotland, Sweden and Ireland are observed to form a well-supported subclade within the genus (Clade L, Figures 8, 9, and 10). For the North American subclade, specimen from British Columbia, Oregon, Ontario, Newfoundland, Nova Scotia and New Hampshire are observed to form a well-supported cluster. The similarity of haplotypes seen among members of each continent and the low observed variation between the two continents could possibly be attributed to the specimens coming from a single biome (costal plains) or the lack of genetic divergence over a long period of time. The latter seems more plausible given the geological history of these costal areas and the parallel zoogeographic trend observed in some fish (Riggs, 1984; Hocutt *et al.*, 1986). This biogeographic trend cannot be establish in the COI gene sequence analyses since the genus *Lemanea* is represented by only two taxa, one from each continent (North America and Europe). More specimens from both continents could be included in future COI studies to examine the biogeography. The genus *Lemanea* (Clade L, Figures 8, 9, and 10) is shown to form a sister taxon with genus *Paralemanea* (Clade K, Figures 8, 9, and 10). This is expected since they belong to the same family, Lemaneaceae.

The two genera *Lemanea* and *Paralemanea* where once classified as subgenera in *Batrachosperum* (Silva, 1959) until Vis and Sheath (1992) raised them to genus level. This elevation to genus-level was confirmed by Sheath *et al.* (1996) based on further research on morphology, ultrastructure and classification of the Lemaneaceae. The genus *Lemanea* is characterized by species that lack axial cortication and the genus *Paralemanea* by species that have axial cortication (Xie *et al.*, 2004; Kučera and

Marvan, 2004). The grouping of *Lemanea* species (Clade G, Figures 8, 9, and 10); however, forms a well-supported sister taxon with *P. annulata* AF029153. Examining *P. annulata* AF029153 sample from GenBank in comparison to *P. annulata* DQ449029 (also included in this study) suggests the placement of *P. annulata* AF029153 is not attributed to sequence artifact (i.e. bad sequence, N's within sequence, etc.) but instead could be due to possible incipient speciation or misidentification. Whether or not there may be any morphological difference between *P. annulata* AF029153 and other *P. annulata* species is the subject of another study. Nonetheless, observation of the *rbc*L gene sequence data suggests the need for additional sampling of genus *Paralemanea* in order to establish intraspecific variation. Clades K (Figures 8, 9, and 10) represent members of genus *Paralemanea* and comprise *P. catenata* and *P. annulata* species. This clade is well-supported with two subclades easily identifiable. The first subclade is well-supported and groups the species *P. annulata*. The second subclade is also well-supported and groups specimens of *P. catenata* with a *Paralemanea* sp. from Kenya, Africa. The same grouping of the *Paralemanea* sp. from Kenya with *P. catenata* is observed in the COI sequence analyses. Coherence between the two genes (COI and *rbc*L) sequence data suggest the *Paralemanea* sp. from Kenya could probably constitute another lineage of *Paralemanea,* and while it is not grouping directly with *P. catenata* in both gene sequence analyses, it is worthwhile to note that almost all *P. catenata* species employed in both gene sequence analyses are from California, USA. Consequently, these localized specimens appear to be genetically identical, hence the coherent grouping from the Kenyan sample. All the above sections and genera described falls under the order Batrachospermales.

4.2 INFRAGENERIC-LEVEL TAXONOMY

At the infrageneric level, seven out of the eight sections of the genus *Batrachospermum* can be clearly distinguished using both COI and *rbc*L genes sequence data. The section *Batrachospermum* is observed to be both cryptic and paraphyletic for both COI and *rbc*L genes sequence data analyses (Clade G and Clade K, Figures 4, 5, 6, 7; Clade H and Clade I, Figures 8, 9, 10). Members of section *Batrachospermum* are split into two clades with Clade K (Figures 4, 5, 6, 7) and Clade I (Figures 8, 9, 10) comprising exclusively of *B. gelatinosum* and Clade G (Figures 4, 5, 6, 7) and Clade H (Figures 8, 9, 10) made up of *B. heterocorticum, B. boryanum,* and *B. involutum*. This split suggests that perhaps section *Batrachosspermum* be split into two separate sections to represent the spit, with one section being monotypic, represented by the species *B. gelatinosum.*In the present study, in the COI gene sequence data, the cryptic section *Batrachospermum* is paraphyletic since is shown to form a sister taxon with the family Lemaneaceae, genera *Sirodotia* and *Tuomeya*. A noteable pattern is also observed in the *rbc*L gene sequence data. These suggest again that the taxonomic ranking of all members of the Batrachospermales, especially genus *Batrachospermum* be re-examined. Confirmation of the cryptic diversity of section *Batrachospermum* by COI is is no surprise since COI has been recognized to reveal cryptic diversity in groups normally not revealed by other markers (e.g. Hebert, Penton *et al.*, 2004; Hebert, Stoeckle, *et al.*, 2004; Ward *et al.*, 2005; Smith *et al.*, 2006; Hajibabaei *et al.*, 2006; Robba *et al.*, 2006). For example, Robba *et al.*(2006), study of the red algae using COI revealed cryptic diversity in *Bangia fuscopurpurea, Corallina officinalis, G. gracilis, M. stellatus, Porphyra leucosticta* and *P.umbilicalis*. Comparison of the COI with the *rbc*L by the authors revealed the COI was more sensitive in revealing incipient or cryptic diversity. This has also been seen in other organisms, for example, Hebert, Penton *et al.* (2004) study of neotropical skipper butterfly *Astraptes fulgerator* using COI gene sequence data together with morphological characters revealed at least 10 species in this butterfly. In a study by Vis and Sheath (1998) examining the molecular and morphological relationship between the two section *Batrachospermum* species (*B. gelatinosum* and *B. spermatoinvolucrum*) using the rDNA ITS 1 and 2 spacer and *rbc*L gene

sequence data, in addition to morphometric characters lead to the reduction of *B. spermatoinvolucrum* to a form of *B. gelatinosum*. Although morphological character like spermantia bearing distinguishes *B. spermatoinvolucrum* from *B. gelatinosum,* and confirmed by morphometric data, sequence divergence from molecular analyses indicate the two species are identical. Thus, there appears to be little congruence between morphology and molecular analyses. The morphological characters (i.e., straight undifferentiated carpogonial branches; well-developed whorls; carpogonia with trichogynes (club- to urn- shaped) (Kumano 1993; 2002; Vis *et al.*, 1995) used to distinguish this section, are insufficient and more characters need to be suggested to discriminate members that possess these features. Like Vis and Sheath (1998), a similar study investigated *B. gelatinosum* intraspecific variation in North America using the ribosomal internal transcribed spacer (ITS) and this marker provided little variation across a large geographic region (Vis and Sheath, 1997). Consequently, it is clear that of the two methods (morphology vs. molecular), molecular can best resolve species relationships in this section.

The Section *Contorta* represented by the species *B. ambiguum, B. globosporum, B. louisanae, B. intortum, B. procarpum* and *B. confusum* (COI only), forms a well-supported clade in all analyses of both the COI and *rbc*L genes (Clade A, Figures 5, 6, and 7; Clade E, Figures 8, 9 and 10). Nonetheless, in the COI analyses, *B. breutelii* which is the monotypic species in the section *Gonimopropagulum* is observed to cluster within the section *Contorta*, whereas in the *rbc*L analyses, section *Gonimopropagulum* is placed as a separate branch (Figures 8, 9 and 10). This latter relationship is in accord with other *rbc*L gene sequence studies (e.g. Müller *et al.*, unpublished) and morphological classification (e.g. Sheath and Whittick, 1995). Previously classified under the section *Aristata*, *B. breutelii* was recognized to belong in a separate section (section *Gonimopropagulum*) because it possessed distinct gonimoblast propagules **(**Sheath and Whittick, 1995**)**. Interestingly, while section *Gonimopropagulum* does not group within the section *Contorta* as observed in the COI gene sequence analyses (Figures 5, 6 and 7), in Müller *et al.*(unpublished) section *Gonimopropagulum* is placed on a separate branch. This discrepancy between the COI and *rbc*L genes sequence data could be attributed to

again the simple concept that DNA barcoding is mainly for purposes of identification and clustering and not to infer species relationships. Thus, DNA barcoding is appropriate for differentiation at the species-level and not higher taxonomic levels. In a study by Vis and Entwisle (2000), examining the phylogeny of Batrachospermales using *rbc*L gene sequence data of Australian taxa, the authors circumscribed the section *Contorta* to include *Batrachospermum* species with curved or twisted carpogonial branches, which included all species previously classified under section *Hybrida*. In this present study however, no members of section *Hybrida* are included in both gene sequence analyses; thus, the merging of the two sections under section *Contorta* is not supported. Future studies of this section using COI gene sequence data could include previously described members of section *Hybrida* for comparison with the *rbc*L to further establish the relationship between the two sections. In the present study, the section *Contorta*, in the COI gene sequence data is observed to form a sister taxon with all other members of study (family Lemanaeceae; genus: *Thorea, Tuomeya, Sirodotia*; sections: *Virescentia, Turfosa, Aristata, Setacea, Batrachospermum*). Clearly the section *Contorta* is paraphyletic since it does not contain of all its members. This again can be seen in the *rbc*L gene sequence data. Again the taxomonic rankings of these groups need to be examined since a section appears to diverge to contain taxonomic levels (family, genus) that are much higher.

The clade comprising species of section *Aristata* is well-supported in all analyses of COI and *rbc*L sequence data (Clade E, Figures 5, 6, and 7; Clade C, Figures 8, 9, and 10). The species *B. macrosporum* in this section is characterized by straight, long carpogonium-bearing branches that have been differentiated from fascicles (Sheath *et al.*, 1994a; Kumano, 2002). Despite the strong support of this section in all analyses in the present study, the monophyly of this section has been called into question by Vis and Entwisle (2000) due to observed paraphyly of the two species *B. macrosporum* and *B. cayennense* studied. Low support for these two species was also observed by Müller *et al.* (unpublished). While these findings may be accurate, the current study of this section employed only the species *B. macrosporum* and hence cannot substantiate the paraphyly of this section. *Batrachospermum*

macrosporum in North America is observed to occur restricted to streams of the costal plains and tropical areas (Vis and Hodge, 2008). In a recent phylogeographic study by Vis and Hodge (2008) of *B. macrosporum* from North and South America using the mitochondrial intergenic spacer between the cytochrome oxidase subunit 2 and 3 *(cox2-3)* revealed splits between the North and South America haplotypes with confirmation from *rbc*L gene sequence data. In addition, Vis and Hodge (2008) observed very little genetic variation among the North American haplotypes and in contrast high variation among haplotypes from South American locations. The author's theorized that variation among North American haplotypes could be attributed to a fairly recent colonization event along the coastal plains. Variation in South American haplotypes on the other hand was hypothesized to be due to the Amazon region serving as a center for diversity. In this present study however, no biogeographic trend is observed for this section since very few specimens of its members were used in the *rbc*L gene analyses and although sufficient specimens were employed in the COI sequence analyses, almost all the specimen are of North American origin. More specimens from distant locations would be needed to ascertain any biogeographic pattern in this section. In the preent study, this section is observed to form a sister taxon with the genus *Thorea* in the COI sequence data. Nonetheless, in the *rbc*L gene sequence data it is observed to form a sister taxon with section *Contorta*, both of which form a sister taxon with a cluster that diverge to form the the family Lemanacea, sections *Virescentia, Gonimopropagulum, Setacea, Batrachospermum,*and genus *Sirodotia.* Again results of both gene sequences raising the question of the validity of taxonomic ranking employed for these groups, whether it be section, genus or even family.

The section *Virescentia* is observed to form a well-supported clade in both COI and *rbc*L sequence data analyses and is represented *B. helminthosum* and *B. virgatum* (Clade B, Figures 4, 5, 6, and 7; Clade D, Figure 8, 9, and 10). Morphologically, species within this section are differentiated by whether they are monoecious or dioecious, have a shade of green, localized, and length of carpogonial branch (Mori, 1975; Starmach, 1977). Previous study on the distribution and systematics of this section in North America by Sheath and Vis (1994) using multivariate morphometrics and image analysis revealed

that the qualitative features (e.g. pigmentation, shape of whorl, shape of carposporophyte etc) used to separate species in this section are not taxonomically useful since they appeared to be present universally. As well, quantitative characters (e.g. whole diameter, fascicle length etc) observed, appeared to be highly variable and overlapped among examined specimens. Biogeographic patterns observed for species of this section have been described as distinct (Chiasson *et al.*, 2003; Hanyuda *et al.*, 2004) and in North America, have been reported to occur widely in eastern USA (Flint, 1948; 1050; Moul and Buell; 1979; Sheath and Burkholder; 1985; Sheath and Cole, 1993). In western North America, this section has been reported in Oregon (Sheath *et al.*, 1986) and Washington (Sheath and Hambrook, 1988). In the study by Hanyuda *et al.* (2004), examining the biogeography and taxonomy of *B. helminthosum* in Japan, including North American haplotypes using the plastid *rbc*L gene, revealed clear clustering of the Japan haplotypes from the North American haplotypes. This difference in haplotype between the Japan and North American specimen was noted by Hanyuda *et al.* (2004) to be potentially attributed to continental drifts and the consequent isolation in the Mesozoic era. Nevertheless, in this present study, no apparent evidence of biogeographic trend is observed for the members of section *Virescentia*, although specimens used ranged from North America to Africa. Hence, more specimens of section *Virescentia* would be needed from both continents to discern any biogeographic patterns. In the present study, both COI and *rbc*L genes sequence suggest the section *Virescentia* is paraphyletic since both genes indicate other sections, genera and family diverge off this section.

Section *Turfosa*, represented by *B. turfosum* Clade C (Figures 5, 6 and 7) and Clade B (Figures 8, 9 and 10) is a well-supported clade in both the COI and *rbc*L gene sequence analyses. This section previously contained *B. turfosum* and *B. keratophytum* that were distinguished from each other based on whether they were carposporic or monoporic, however, studies by Müller *et al.* (1997) concluded that *B. keratophytum* and *B. turfosum* are synonymous based on small sequence divergence between ITS1 and 2 regions of the two species and that monosporic plants (previously only noted in *B. turfosum*) could be observed in *B. keratophytum* at different times of the year. In the present study, the proportion of sites

differing (0/572) for the COI gene may suggest that the six samples analyzed in this study all belong to the species *B. turfosum*. Morphologically, *B. turfosum* is observed to possess straight, short, differentiated carpogonial branches, characteristics which are also observed in members of the section *Virescentia*. Nonetheless, like this study and other molecular studies (e.g. Vis *et al.*, 2005) these two sections appear autonomous and the present study, appear in separate clades although not that distant from each other in the COI and *rbc*L sequence data analyses phylogenies (Figures 5, 6, 7, 8, 9 and 10). In North America, members of section *Turfosa* are observed to occur in ponds, pools and bogs (Flint, 1957; Yung *et al.*, 1986; Wehr and Sheath, 2003) and recognized as the third most widely distributed species of freshwater rhodophyte in North America (Sheath and Cole, 1992; Sheath *et al.*, 1994). Again, like the section *Virescentia*, no biogeographic trend is apparent in both the COI and *rbc*L genes sequence analyses (Clade B, Figures 4, 5, 6 and 7; Clade D, Figures 8, 9, and 10). More specimens of members of section *Turfosa* from distant locations would be need to establish any biogeographic patterns. Like other sections shown above, the section *Turfosa* is paraphyletic and forms a sister taxon with a cluster that contains other section, genera and family, as shown by both COI and *rbc*L gene sequence data in the present study.

The final section, *Setacea* is shown to be well-supported in both COI and *rbc*L gene analyses. In the *rbc*L analyses Clade G (Figures 8, 9, and 10) this section is observed to be monophyletic, however in the COI analyses Clade F (Figures 5, 6, and 7) members do not form a monophyletic cluster. The species *B. atrum* (BI13) is observed to be placed on a separate branch, distant from the section *Setacea* clade (Clade F, Figures 5, 6, and 7). This section in the COI analyses is shown to form a sister taxa with section *Batrachospermum* (Clade G, Figures 5, 6 and 7). Based on the presence of well-developed whorls and reduced whorls, members of the section *Setacea* were suggested by Necchi (1990), Necchi and Entwisle (1990) and Vis and Entwisle (2000) to be integrated into section *Virescentia*, however, looking at the phylogeny for both COI and *rbc*L gene sequence data, the two sections; *Setacea* and *Virescentia* are distant from each other, suggesting the rejection of integration of the two sections based on morphometric

data by Sheath *et al.* (1994a) was valid. While members within this section do not form a complete coherent cluster in the COI gene sequence data, with the omission of *B. atrum* BI13, analyses of members of section *Setacea* suggest possible geographic patterns in this taxon. Samples from the same geographic region, Scotland and England (0.00% COI sequence variation) appear to be the same species, *B. atrum* in the COI genes sequence data. The specimen from Kenya, a relatively distant location from Scotland and England, was observed to substantially differ in COI sequence variation, 8.5% from the Scotland and England samples. Should the Kenyan specimen be identified as *B. atrum*, then the difference in sequence divergence between the two geographic locations would suggest a much longer divergence time of the Kenyan sample from the Scotland and England samples.

4.3 GENERAL CONCLUSION

Prior to the impact of molecular data, ordinal classification of members of the red algae depended predominantly on analyses of female reproductive structures before and after fertilization. The application of molecular methods to systematics has further improved our understanding of the red algae at the different taxonomic levels as well as led to the recognition of new orders. Over the past two decades, relatively few molecular markers have been used in studies of the red algae systematics. Molecular markers have proven to be useful, not only in elucidating red algae systematics but also in discovering genetic variation within red algae species. A recent shift is in the use of DNA sequences as a tool for identification, such as DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. Through sequence data and phylogenetic sequence analysis, it is become apparent that the COI can be used in identifying and clarifying species relationships in the red algae (Rhodophyta) (e.g. Saunders, 2005; Robba *et al.* 2006, House *et al.* (2008). Based on this, the present study was initiated to address the taxonomic

issues among the freshwater red algal orders Batrachospermales and Thoreales using the DNA barcoding protocol and evaluating the following

a) the efficacy of the DNA barcoding protocol in resolving species relationships in the Batrachospermales and Thoreales

b) the usefulness of the cytochrome c oxidase subunit I (COI) in identifying interspecific and intraspecific variations in members of the Batrachospermales and Thoreales

c) any possible geographic variation in members of the two orders and

d) compare results of the COI gene to that of the plastid *rbc*L gene.

The effectiveness of the DNA barcoding protocol in resolving species relationships in the two orders Batrachospermales and Thoreales as it relates to the initial objective (a, above) of the present study was clearly established. Not only was DNA barcoding able to resolve sepecies level relationships but was also efficient at identifying different sections, genera and even family. In the present study, distinct grouping of geographically consistent specimens were observed. This could be seen in all seven sections (*Aristata, Batrachospermum, Setacea, Contorta, Gonimopropagulum* and *Virescentia*) of the paraphyletic genus *Batrachospermum*. Relations of these sections are however not clearly defined. The monotypic section *Gonimopropagulum* is shown to group within the section *Contorta*, further re-enforcing that DNA barcoding is not meant to and does not provide evolutionary information about taxa. At the generic level, *Tuomeya*, *Sirodotia, Lemanea, Paralemanea,* and *Thorea* are clearly identified. The relationship between *Lemanea* and *Paralemanea* can be clearly seen as sister taxon forming the family Lemanaeceae. At the ordinal level; however, the relationship between Batrachospermales and Thoreales is not resolved.

Through COI gene sequence data anylyses, the Thoreales is shown to clade within the Batrachospermales, which is contradictory to observations by Entwisle and Necchi (1992), Müller *et al.* (2002), and *rbc*L gene sequence results of the present study. Consequently the barcoding protocol was successful at forming distinct groupings at both the sectional, generic level and family level that were stable and sufficiently separated from other such groups such that taxonomic inferences could be made. Nonetheless, it was unsuccessfully in resolving species relationships in the Batrachospermales and Thoreales.

In the second objective, the COI gene in fact did prove useful in identifying interspecific and intraspecific variations in members of the Batrachospermales and Thoreales. This verification of the COI has already been evaluated in other member of the red algae (e.g. Saunders, 2005; Robba *et al.*, 2006; House *et al.*, 2008) but not in the freshwater Thoreales and majority of the Batrachospermales members employed in this study. Saunders *et al.* (2005), Robba *et al.* (2006) and House *et al.* (2008) evaluation of the COI in the red algae all indicated the COI was variable within red algal populations and suggested species delimitation using the COI gene. Results of the present study support the use of the COI gene to identify inter- and intraspecific variation in the red algae (Batrachospermales and Thoreales). This is clearly evident in the present study, in the UPGMA analysis where intraspecific variation of 0 to 15 bp (2.3%) is observed forms a unique isolate of *B. gelatinosum* (Clade E, Figure 4). Interspecific variation ranged from 58 bp (9.03%) (between *B. procarpum* and *B. intortum*) to 78 bp (12.15%) (between *B. ambiguum* and *B. globosporum*). Members of the order Thoreales differed intraspecifically by an average of 81 bp Clade L (Figure 4)**.** As expected**,** interspecific variation is higher than intraspecific variation. The high intraspecific divergence values for *B. macrosporum* and *B. gelatinosum* and the grouping of these sequences into multiple biogeographic lineages raise several possibilities, including the presence of multiple species, cryptic species or incipient species. Given that no characters could be found to separate these groups using standard taxonomic characters for the genus *Batrachospermum*, it would not be sensible to describe new taxa based primarily on these short DNA sequences. Although the clusters may represent cryptic or incipient species, as asserted for section *Batrachospermum*, telling apart these

phenomena is impossible with the present dataset. Additionally, *B gelatinosum* and *B. macrosporum*, which had the highest intraspecific divergences, are also the two taxa for which the greatest numbers of accessions were included in the analyses. Similar divergence levels may be present in other batrachospermalean taxa, and more studies would need to be undertaken to examine other species in a similar or greater depth (i.e. sequencing and morphological analysis of many representatives across the geographic range of the taxon) to be able to expand generalizations about intra versus interspecific sequences divergences using DNA barcoding in this order.

The North American biogeographic trends of members of the freshwater order Batrachospermales have been studied using both molecular and morphological data (e.g. Vis and Sheath, 1997; Vis *et al.*, 2008). For example Vis *et al.* (2001) investigated the biogeography of *B. helminthosum* in North America, both molecular and morphological variation. The chloroplast *rbc*L gene and the nuclear ribosomal ITS regions were sequenced, and 5 *rbc*L haplotypes and 11 ITS genotypes were revealed. The phylogeography of this species was concluded to be complex, with samples from distant locations being genetically similar. However, present research on this order using COI gene sequence data suggests that this marker is appropriate for phylogeographic studies (House *et al.*, 2008; Sherwood *et al.*, 2008), but unlike Vis *et al.* (2001), hints of biogeographic trends was evident in this study for both the COI and *rbc*L sequence data; however a lot more in the *rbc*L gene. Samples of certain specimen from remote regions (North America, Europe and Africa) did appear genetically identical in this study for both COI and *rbc*L genes sequence data. Identical genetic composition of the samples could suggest the samples potentially originated from similar locations. While biogeographic trends have been reported in members of the Batrachospermales in several studies using the *rbc*L gene (e.g. Vis *et al.*, 1998; Vis and Entwisle, 2000; Vis *et al.*, 2001; Hanyuda *et al.*, 2004; Vis *et al.*, 2005), especially in members of the genus *Batrachospermum*. In the present study for the *rbc*L gene, biogeographic relationships were evident in the phylogenetic analyses for some groups although more specimens would be needed to establish such trend in the remaining groups (e.g. Clade F and G, Figures 8, 9, and 10). The COI gene to a certain extent did

establish biogeographic patterns in some groups although, more samples from North America, as well as Europe and Africa would be needed to fully establish any pattern. For the COI gene the strongest biogeographic trend is shown in *B. atrum* Clade I (Figures 5, 6, and 7) and the characteristics of this trend may be demonstrated in all North American freshwater algae, or perhaps only in this species. More research and specimens from distant geographic locations would need to be included to examine and ascertain any patterns in the freshwater rhodophytes of North America. For the order Thoreales, very few samples are represented in this study and no biogeographic trends is apparent in neither the *rbc*L nor the COI gene sequence data analyses. Again, more samples from remote geographic locations would be needed to establish any biogeographic patterns in this order.

Observations from both COI and *rbc*L genes sequence data clearly suggest the current taxonomy of the Batrachospermales need to be re-examined. The validity of section level classification is strongly questioned in the present study, since several sections in both COI and *rbc*L gene analyses are observed to form sister taxa with different genera. The genus *Batrachospermum* is notably made up of species that are similar in overall morphology, but genetically divergent (Vis *et al.*, 1998). The recognition of sections under the *Batrachospermum* have been based primarily on the shape and position of carpogonial branch, which carry the carpogonium and is made up often of cells that are differentiated or specialized. These infrageneric sections were used to delineate northern hemisphere taxa, mainly of European origin. As noted by Kumano (1993) the sectional classification has undergone numerous revisions and may change with further systematic research. The present study can be considered as one such systematic study. Results of both COI and *rbc*L gene sequence indicate the taxonomy of the genus *Batrachospermum* be revised. Based on the recent elevation of the Thorea from genus to ordinal status, the following proposals are made:

a) Elevate the sections *Virescentia, Aristata, Contorta, Turfosa* and *Setacea* all to the level of genus. This would resolve the issues of genus and families branching off sections. This would eliminate

the current genus *Batrachospermum* and recognize the family Batrachospermaecea with more than the current four genera.

b) Due to the cryptic diversity of the section *Batrachospermum*, we propose it be elevated to genus status but split into two genera with the first maintaining the name 'genus *Batrachospermum'* as a monospecific genus with the type species *B. gelatinousm*. The second genus could perhaps be recognized as 'genus *pseudogelatinosum*' comprising the remained of species currently recognized under the section *Batrachospermum* (*B. anatinum B. arcuatum B. boryanum B. confusum B. heterocorticum B. involutum B. spermatoinvolucrum B. sporulan*. This proposal is however, not definitive since the present study included only *B. heterocorticum B. involutum* and *B. boryanum* in both COI and *rbc*L gene sequence data. It can however be postulated based on sequence divergence observed for members of section *Batrospermum*, that species of the section *Batrachospermum* not included in the present study would group under the newly proposed 'genus *pseudogelationsum*'.

c) The current monotypic section *Gonimopropagulum* be elevated to a monospecific genus with the type species *B. breutelii*. Although results of the COI and *rbc*L genes sequence are incongruent in the present study, it is clear *B. breutelii* warrant its own ranking and should all the current classified sections be raised to the status of genus, then section *Gonimopropagulum* merit such an elevation too.

d) Maintain the genus *Sirodotia, Tuomeya, Lemanea* and *Paralemanea*

e) Maintain the family Lemaneaceae

f) Maintain the orders Batrachospermales and Thoreales

In summary, the present study has contrasted the utility of a short organellar marker for construction of DNA barcode-like data frameworks for two orders of red algae, and has compared these results to the plastid *rbc*L gene from this study and other previously published analyses. These results provide the first DNA barcode data for the order Thoreales to my knowledge. The present study has illustrated the utility of DNA barcodes for highlighting taxonomic and potential biogeographic trends within the Batrachospermales, and demonstrates that red algal intraspecific divergence values can be much higher than previously reported. DNA barcoding, together with more genetic markers and traditional analyses, will play an important role in species level taxonomic studies of the Batrachospermales in the future.

REFERENCES:

Adam Z, Zaltsman A, Sinvany-Villalobo G, Sakamoto W.2005.FtsH proteases in chloroplasts and cyanobacteria Physiologia Plantarum, 123: 386 - 390

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A. *et al.*, (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52, 399–451.

Andersen RA.1992. Diversity of eukaryotic algae. Biodivers. Conserv., 1 : 267–292.

Atkinson, K.M.1980. Experiments in dispersal of phytoplankton by ducks. Br. Phycol. J. 15: 49 – 58

Babu, B. AND. Baluswami M. 2005. Tuomeya americana (Kuetzing) Papenfuss,a fresh-water red alga, new to India. Indian *Hydrobiol*. 8: 1–4.

Branco, LHZ and Necchi Jr, O. 1997. Seasonality of macroalgae in three tropical drainage basins in São Paulo State, southeastern Brazil. Archiv für Hydrobiologie 141:75-91

Brodie J, Hayes PK, Barker GL, Irvine LM, Bartsch I.1998. A Reapraisal of Porphyra and Bangia (Bangiophycidae, Rhodophyta) in the Northeast Atlantic based on the *rbc*L-*rbc*S intergenic spacer. Journal of Phycology, 34:1069–1074.

Borge, O. 1928. Su¨ sswasseralgen. Hedwigia 68: 93–114, 2 pls.

Butterfield NJ.1990.Organic Preservation of Non-Mineralizing Organisms and the Taphonomy of the Burgess Shale Paleobiology, 16: 272-286

Butterfield NJ.2000. Bangiomorpha pubescens n. gen., n. sp.: implications for the evolution of sex, multicellularity, and the Mesoproterozoic/Neoproterozoic radiation of eukaryotes. Paleobiology, 26:386–404.

Bolivar I, Fahrni JF, Smirnov A, Pawlowski J.2001. SSU rRNA-based Phylogenetic Position of the Genera Amoeba and Chaos (Lobosea, Gymnamoebia): The Origin of Gymnamoebae Revisited Molecular Biology and Evolution 18:2306-2314

Carmona JJ and Necchi Jr O.2001. Systematics and distribution of *Thorea* (Thoreaceae, Rhodophyta) from central Mexico and south-eastern Brazil. Phycological Research 49: 231–239

Chen CA and Yu Jr-Kai.2000.Universal Primers for Amplification of Mitochondrial Small Subunit Ribosomal RNA-Encoding Gene in Scleractinian Corals. Mar. Biotechnol. 2: 146–153

Countway PD, Gast RJ, Savai P, and Caron DA. 2005. Protistan diversity estimates based on 18S rDNA from seawater incubations in the western North Atlantic. *J Eukaryot Microbiol.*, 52: 95 – 106

de Vargas C, L Zaninetti, H Hilbrecht, J Pawlowski1..1997. Phylogeny and Rates of Molecular Evolution of Planktonic Foraminifera: SSU rDNA Sequences Compared to the Fossil Record. J Mol Evol, 45:285–294

Dawkins R. 2004. The Ancestors Tale. Phoenix, London.

Ehara, M., Y. Imaga, K. Kazuo, I. Watanabe and T. Ohama. 2000. Phylogenetic analysis of diatom coxI genes and implications of a fluctuating GC content on mitochondrial code evolution. Curr. Genet. 37: 29-33

Eloranta P. AND Kwandrans J. .1996. Distribution and ecology of freshwater red algae (Rhodophyta) in some central Finnish rivers. *Nordic J. Bot*. 16: 107–117.

Entwisle TJ and Kraft GT. 1984. Survey of freshwater red algae (Rhodophyta) of south-eastern Australia. Aust. J. Mar. Freshw. Res. 35: 213 – 59

Entwisle TJ, Vis ML and Foard HJ. 2000. Biogeography of Psilosiphon (Batrachospermales, Rhodophyta) in Australia and New Zealand. Cryptog. Algol. 21: 133 – 48

Entwisle, T.J. & Foard, H.J. (2007). Algae of Australia Batrachospermales, Thoreales, Oedogoniales and Zygnemaceae. (Entwisle, T.J., Skinner, S., Lewis, S.H. & Foard, H.J. Eds), pp. 1-25. Canberra & Melbourne: Australian Biological Resources Study & CSIRO Publishing

Feng, J., XIE S-L., AND. YAO G. 2007. Tuomeya Harvey, a genus of freshwater Rhodophyta newly recorded from China. *Acta Phytotax. Sin*. 45: 719–726.

Flint, LH.1948. Studies of freshwater red algae. Am.]. BoG 35: 428 - 433

Flint, LH.1953. Two new species of *Batrachospermum*. Proc. La. Acad. Sci. 16: 10 – 15

Flint LH. 1957. Notes on algae of Quebec. II. Laurentide Park. *Naturalise Canadien*, 84: 179 – 181

Flint LH. 1970. *Freshwater Red Algae of Norht America*. New York: Vantage

Fredericq S and Ramírez ME. 1996. Systematic studies of the Antarctic species of the Phyllophoraceae (Gigartinales, Rhodophyta) based on *rbc*L sequence analysis. Hydrobiologia 326/3277: 137 – 43

Freshwater, D. W., Fredericq, S., Butler, B. S., Hommersand, M. H. & Chase, M. W. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. Proc. Natl. Acad. Sci. USA 91:7281–5.

Freshwater DW, Fredericq S and Hommersand MH. 1995. A molecular phylogeny of the Gelidiales (Rhodophyta) based on analysis of plastid *rbc*L nucleotide sequence. J. Phycol. 31: 616 – 32

Freshwater DW, Fredericq S and Bailey JC. 1999. Characteristics and Utility of nuclear-encoded large-subunit ribosomal gene sequences in phylogenetic studies of red algae. Phycological Research 47: 33 – 38

Gabrielson P. W., D. J. Garbary & R. F. Scagel, 1985. The nature of the ancestral red alga: inferences from cladistic analysis.Biosystems 18: 335–346.

Gabrielson PW and Garbary D (1986) Systematics of red algae (Rhodophyta). CRC Critic Rev Plant Sci 3:325-36

Gabrielson PW, Garbary, Sommerfeld MR, Townsend RA, Tyler PL.1990. Phylum Rhodophyta. In L. Margulis, J. O. Corliss, M. Melkonian, and D. J. Chapman [eds.], Handbook of protoctista, 102–118. Jones and Bartlett, Boston, Massachusetts, USA

Garybeal A. 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? System Biol. 47: 9 – 17

Glimn-Lacy J and Kaufman, PB.1984.Botany Illustrated. Kluwer Academic Publishers

Goldberg A, Wildman DE, Schmidt TR, Hutteann M, Goodman M, Weiss ML Grossman LI. 2003. Adaptive evolution of cytochrome c oxidase subunit VIII in anthropoid primates. National Academy of Sciences, 100. 5873 - 5878

Gordon RB. Bulletin of the Torrey Botanical Club, Vol. 61, No. 8 (Nov., 1934), pp. 437-439

Han D, Morgan JE, Gennis RB.2005. G204D, a Mutation That Blocks the Proton-Conducting D-Channel of the aa3-Type Cytochrome c Oxidase from Rhodobacter sphaeroides. American Chemical Society, 44. 12767 - 12774

Hajibabaei M, Janzen DN, Burns JM, Hallwachs W, Hebert PDN. 2006. DNA barcodes distinguish species of tropical Lepidoptera. *Proc Natl Acad Sci USA*, 103: 968 – 971

Hajibabaei M, Singer GAC, Hebert PDN and Hickey DA. 2007. DNA barcoding: How it compliments taxonomy, molecular phylogenetics and population genetics. *Trends Genet.*

Harper JT and Saunders GW.2001. Molecular systematics of the Florideophyceae (Rhodophyta) using nuclear large and small subunit rDNA sequence data. Journal of Phycology, 37: 1073 – 1082.

Harvey WH. 1858. Nereis Boreali-Americana. Part III─Chlorospermae. Washington, DC: Smithsonian Contributions to Knowledge. 138.

Hebert PDN, Cywinska, A., Ball SL ,DeWaard JR.2003a. Biological identifications through DNA barcodes. Proc. R. Soc. B 270: 313–322.

Hebert, PDN., Ratnasingham, S. & deWaard, J. R. 2003b.Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. B 270: S96 – S99.

Hebert PDN, Penton EH, Burns JM, Janzen DH and Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc Natl Acad Sci USA*, 101: 14812 – 17

Hebert PDN., Stoeckle MY, Zemlak TS, Francis CM. 2004. Identification of birds through DNA barcodes. PLoS Biol. 2: 1657–1663.

Hogg ID, Hebert PDN. 2004. Biological Identification of springtails (Collembola: Hexapoda) from the Canadian Artic, using mitochondrial DNA barcodes. *Can J Zool.*, 82: 749 – 754

Hurst GDD, Jiggins FM. 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc. R. Soc. B 272: 1525 – 1535.

Israelson, G.1942. The freshwater Florideae of Sweden. Studies on their taxonomy, ecology, and distribution. Symb. Bot. Ups. 6: 1 – 135

Jiménez, J.C. & Necchi, O., Jr (2001). A new species and expanded distributions of freshwater Audouinella (Acrochaetiaceae, Rhodophyta) from central Mexico and south eastern Brazil. European Journal of Phycology 36: 217-226.

John, D.M., Whitton, B.A. and Brook, A.J. 2002. The Freshwater Algae Flora of the British Isles. Cambridge University Press, Cambridge.

Kapraun, D.F., Braly, K.S. & Freshwater, D.W. (2007). Nuclear DNA content variation in the freshwater red algal orders Batrachospermales and Thoreales (Florideophyceae, Nemaliophycidae). Phycologia 49: 54-62

Kaczmarczyk, D., Sheath, R. G. & Cole, K. M. 1992. Distribution and systematics of the freshwater genus *Tuomeya* (Rhodophyta, Batrachospermaceae). J. Phycol. 28:850–5.

Knoll AH. The early evolution of eukaryotes: a geological perspective. Science 1992; 256: 622 - 627.

Krzywinski, J, Grushiko, OG, and Besansky, NJ.2006. Analysis of the complete mitochondrial DNA from Anopheles funestus: an improved dipteran mitochondrial genome annotation and a temporal dimension of mosquito evolution. Mol Phylogenet Evol 39:417-423.

Kučera P. & Marvan P. (2004): Taxonomy and distribution of *Lemanea* and *Paralemanea* (Lemaneaceae, Rhodophyta) in the Czech Republic. – Preslia, Praha, 76: 163–174.

Kumano, S.1982c. Development of carpogonium and taxonomy of six species of the genus *Sirodotia*. Rhodophyta, from Japan and West Malaysia. Bot. Mag. Tokyo, 95: 125 – 137.

Kumano, S. 1990. Carpogonium and carposporophytes of Menagne's taxa of *Batrachospermum* (Rhodophyta) from French Guiana. Cryptog. Algol., 11: 281 – 292

Kumano, S. 1993. Taxonomy of the family Batrachospermaceae (Batrachospermales, Rhodophyta). Jpn. J. Phycol. (Soˆrui) 41:253–74.

Kumano, S. (2002). Freshwater Red Algae of the World. Biopress Ltd., Bristol.

Kylin, H. 1912. Studien über die schwedischen Arten der Gattungen *Batrachospermum* Roth und *Sirodotia* nov. Gen. Nova Acta Regiae Soc. Sci. Upsal., Series 4, 3: 40 pp

Lara E, Heger TJ, Mitchell EAD, Meisterfeld R, Ekelund F.2007. SSU rRNA Reveals a Sequential Increase in Shel Complexity Among the Euglyphid TestateAmoebae (Rhizaria: Euglyphida) Protist, 158: 229—237

Lee, RE.1971. The pit connections of some lower red algae: ultrastructure and phylogenetic significance. Br. Phycol. J. 6: 29– 38.

Le Gall L and Saunders GW.2007. A nuclear phylogeny of the Florideophyceae (Rhodophyta) inferred from combined EF2, small subunit and large subunit ribosomal DNA: establishing the new red algal subclass Corallinophycidae. Mol Phylogenet Evol, 43: 1118 – 30

Litaker RW, Vandersea MW and Kibler SR *et al.*2007. Recognizing dinoflagellates species using ITS rDNA sequences. J. Phycol., 43: 344 – 355.

Magne F. 1967. Sur le deroulement et le lieu de la meiose chez Lemaneacées (Rhodophycées, Nemalionales. C. R. Acad. Sci. (Paris), sér. D 265: 670 – 3

Merzlyak E, Yurchenko V, Kolesnikov AA, Alexandrov K, Podlipaev SA, and Maslov DA.2001.Diversity and Phylogeny of Insect Trypanosomatids Based on Small Subunit rRNA Genes: Polyphyly of Leptomonas and Blastocrithidia. The Journal of Eukaryotic Microbiology, 48: 161 – 169

Mori, M. 1975. Studies of the genus *Batrachospermum* in Japan. Jap. J. Bot. 20:461–84.

Müller, KM, Vis, ML, Chiasson, WB, Whittick,A, Sheath, RG.1997. Phenology of a Batachospermum populations in a boreal pond and it implications for the systematics of section *Turfosa* (Batrachospermales, Rhodophyta). Phycologia, 36: 68 – 75

Müller, K.M., Sheath, R.G., Vis, M.L., Crease, T.J. & Cole, K.M. 1998. Biogeography and systematics of Bangia (Bangiales, Rhodophyta) based on the RuBisCO spacer, *rbc*L gene and 18S rRNA gene sequences and morphometric analyses. I. North America. Phycologia 37:1.

Müller, K.M., Sherwood, A.R., Pueschel, C.M., Gutell, R.R. & Sheath, R.G. 2002. A proposal for a new red algal order, the Thoreales. Journal of Phycology 38: 807-820

Müller, K.M., Cannone, J.J. & Sheath, R.G. 2005. A molecular phylogenetic analysis of the Bangiales (Rhodophyta) and description of a new species and genus, Pseudobangia kaycolea. Phycologia. 44: 146-155.

Müller KM, Couture DT, Lynch MDJ and Sheath RG.2008 (submitted).

phylogeny and carpogonial branch differentiation in the freshwater red algal genus *Batrachospermum* (Batrachospermales)

Necchi O Jr. 1984. Catalogo das Rhodophyta de agues continent do Brasil. *Rickia* 11: 99 – 107

Necchi, O Jr. and Branco CCZ.1999. Phenology of a dioecious population of *Batrachospermum* delicatulum Batrachospermales, Rhodophyta) in a stream from southern Brazil. Phycol. Res. 47: 251 – 256

Necchi, O. Jr, Sheath, R. G. and Cole, K. M. 1993. Distribution and systematics of the freshwater genus *Sirodotia* Ecology and morphology of *Sirodotia* huillensis (Batrachospermales, Rhodophyta) in North America. J. Phycol. 29: 236–43.

Necchi, O. JR (1990). Revision of the genus *Batrachospermum* Roth (Rhodophyta, Batrachospermales) in Brazil. Bibl. Phycol., 84: III -}-201 pp., 264 fig.

Necchi, O., Jr. & Entwisle, T. J. 1990. A reappraisal of generic and subgeneric classification in the Batrachospermaceae (Rhodophyta). Phycologia 29:478–488

Necchi, O. Jr. and Zucchi, M.R. (1995) Systematics and distribution of freshwater Audouinella (Acrochaetiaceae, Rhodophyta) in Brazil. Eur .J. Bot. 30:209–218.

Necchi, O. Jr and Zucchi, M. R. 1997. Audouinella macrospore (Acrochaetiaceae, Rhodophyta) is the 'Chantransia' stage of *Batrachospermum* (Batrachospermaceae). Phycologia 36: 220–4.

Orfanidis, S and Breeman AM. 1999.Geographic variation in thermal traits in Digenea simplex and Champia parvula (Rhodophyta) in relation to present and glacial temperature regimes. J. Phycol., 35 : 919–930.

Palmer JD, Soltis DE, Chase MW.2004.The plant tree of life: an overview and some points of view. American Journal of Botany 91 : 1437-1445

Pielou EC. 1979. *Biogeography*. New York: Wiley-Interscience

Pueschel CM and Cole KM.1982. Rhodophycean pit plugs: an ultrastructural survey with taxonomic implications. American Journal of Botany, 69: 703 – 720

Pueschel CM.1989. An expanded survey of the ultrastructure of red algal pit plugs. Journal of Phycology, 25: 625 – 636

Pueschel CM.1994. Systematic significance of the absence of pit-plug cap membranes in the Batrachospermales (Rhodophyta). Journal of Phycology, 30: 310 – 315

Ragan, M.A., C.J. Bird, E.L. Rice, R.R. Gutell, C.A. Murphy and R.K. Singh. 1994. A molecular phylogeny of the marine red algae (Rhodophyta) based on the nuclear small-subunit rRNA gene. Proc. Natl. Acad. Sci. USA 91:7276-7280.

Rajan SS. 2002. Plant Morphology. Anmol Publications Pvt Ltd., New Delhi, Mehra Offset Press, Delhi.

Rantzien HH. 1950. *Tristicha. Najas* and *Sirodotia* in Liberia. *Medelanden fran Göteborgs Botaniska Trädgård* 18: 185 – 197

Ratnasabapaty, M and. Kumano S. 1982. Studies on freshwater red algae of Malaysia. II. Three species of *Batrachospermum* from Sungai Gombak and Sungai Fusu, Selangor, West Malaysia. Jap. J. Phycol. 30: 119-124.

Reis, MP. dos. 1965b. *Batrachospermum* gulbenkianum, sp. nov.. Anuario Soc. Brot., 31: 31 – 44

Reis, MP.1974. Chaves para a identificagao das esp6cies portuguesas de *Batrachospermum* Roth. Ann. Soc. Brot., 40: 37-125.

Ricciardi E.2001. Facultative interactions among aquatic invaders: is an "invasional meltdown" occurring in the Great Lakes? *Can J. Fish Aquat. Sci.*, 58: 1 – 13

Rintoul TC, Sheath RG, Vis ML.1999. Systematics and Biogeography of the Compsogonales (Rhodophyta) with emphasis on the freshwater families in North America. Phycologia, 38: 517 – 527

Robba L, Russel SJ, Barker GL, Brodie J. 2006. Assessing the use of the mitochondrial cox1 marker for the use in DNA barcoding of red algae. American Journal of Botany, 93.1101 – 1108

Roscher, J. 1967. Algal dispersal by muskrat intestinal contents. Trans Am. Microsc. Soc. 86:497-8

Rubinoff D, Cameron S, and Will K. 2006. A Genomic Perspective on the Shortcomings of Mitochondrial DNA for "Barcoding" Identification. *Journal of Heridity*, 97: 581 – 594

Sakaguchi M, Nakayama T, Hashimoto T, Inouye I.2005 Phylogeny of the Centrohelida Inferred from SSU rRNA, Tubulins, and Actin Genes. Journal of Molecular Evolution, 61: 765 – 775

Saunders, GW.1993. Gel purification or red algal genomic DNA: an inexpensive and rapid method for the isolation of polymerase chain reaction-friendly DNA. J. Phycol. 29: 251 – 254.

Saunders GW and Bailey JC.1997. Phylogenesis of pit-plug associated features in the Rhodophyta: inferences from molecular systematics data. Canadian Journal of Botany, 75: 1436 – 1447

Saunders GW and Kraft GT.1997. A molecular perspective on red algal evolution: Focus on the Florideophycidae. The origins of algae and their plastids. Pl. Syst. Evol. Suppl, 11: 115 – 138

Saunders, GW and Hommersand, MH (2004) Assessing red algal supraordinal diversity and taxonomy in the context of contemporary systematic data. Am J Bot 91:1494–1507

Saunders GW. 2005.Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philos Trans R Soc Lond B Biol Sci., 360: 1879 – 1888

Schneider, CW, Wynne, MJ.2007. A synoptic review of the classification of red algal general a half century after Kylin's "Die Gattungen der Rhodophycean." Botanica Marina, 50: 197 - 249

Schnepf E.1992. From prey via endosymbiont to plastid: comparative studies in dinoflagellates. In: Lewin RA (ed) The origins of plastids. Chapman & Hall, New York, pp 53–76

Semyenova SK, Morozova EV, Chrisanfova GG, Gorokhov VV, Arkhipov IA,Moskvin AS, Movsessyan SO, Ryskov AP. 2006. Genetic differentiation in Eastern European and Western Asian populations of the liver fluke, fasciola hepatica, as revealed by mitochondrial NAD1 and COX1 genes. The Journal of Parasitology, 92. 525–530

Sheath, RG. 1984. The biology of freshwater red algae. In Round, F. E. & Chapman, D. J. [Eds.] Progress in Phycological Research, Vol. 3. Biopress, Bristol, pp. 89–158.

Sheath R.G., Vis M.L. and Cole K.M. 1992. Distribution and systematic of Batrachospermurn (Batrachospermales, Rhodophyta) in North America. 1. Section *Contorta*. Journal of Phycoloay 28: 237 – 246

Sheath, RG and Cole, KM.1993. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 2. Chromosome numbers. Phycologia, 32: 304 – 306

Sheath RG, Vis ML, Cole KM.1993a. Distribution and systematics of the freshwater red algal family Thoreaceae in North America. Eur. J. Phycol., 28: 231 – 241

Sheath, R. G., Vis, M. L. & Cole, K. M. 1993c. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 3. Section *Setacea*. J. Phycol. 29: 720–6.

Sheath, R. G., Vis, M. L. & Cole, K. M. 1994a. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 5. Section *Aristata*. Phycologia 34:431–8.

Sheath, R. G., Vis, M. L. & Cole, K. M. 1994b. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 4. Section *Virescentia*. J. Phycol. 30:108–17.

Sheath, R. G., Vis, M. L. & Cole, K. M. 1994c. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 6. Section *Turfosa*. Phycologia 30: 872 – 884.

Sheath RG and Vis ML.1995. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta). Phycologia, 34: 855 – 864

Sheath RG and Whittick A.1995. The unique gominoblast propagulules of *Batrachospermum* bruetelii (Batrachospermales, Rhodophyta). Phycologia, 33: 34 – 38

Sheath, R. G., Müller, K. M., Whittick, A. & Entwisle, T. J. 1996a. A re-examination of the morphology and reproduction of *Nothocladus* lindaueri (Batrachospermales, Rhodophyta). Phycol. Res. 44:1–10.

Sheath, R.G. & Müller, K.M. 1999. Systematic status and phylogenetic relationships of the freshwater genus Balbiania (Rhodophyta). Journal of Phycology. 35: 855-864

Sheath, RG. 2003. Red algae, pp. 197–224. In: J. D. Wehr and R. G. Sheath, eds., Freshwater Algae of North America, Ecology and Classification. Academic Press, San Diego, CA

Shulian X, and Zhixin S.2005. Three new species of *Batrachospermum* Roth (Batrachosper-maceae, Rhodophyta) in China Chinese Journal of Oceanology and Limnology 23: 204-209

Sheath RG. 2006. Biology of Freshwater Algae. Encyclopedia of Life Sciences. John Wiley and Sons, Ltd.

Sherwood AR, Vis ML and Sheath RG. 2004. Phenology and phylogenetic positioning of the Hawaiian endemic freshwater alga,*Batrachospermum spermatiophorum* (Rhodophyta, Batrachospermales). *Phycological Research*, 52: 193 – 203

Sherwood AR., Vis ML., Entwisle TJ., Necchi O, and Presting GG. 2008. Contrasting intra versus interspecies dna sequence variation for representatives of the batrachospermales (rhodophyta): Insights from a dna barcoding approach. *Phycological Research*, 56(4):269-279

Silva PC. 1959. Remark on algol nomenclature, II. *Taxon*, 8: 60 – 64

Sirodot, S. 1873a. Nouvelle classification des algues d'eau douce du genre *Batrachospermum*; Developpement; generations alternanates. Compt. Rend. Acad. Sci. [Paris] 79: 1216 – 1220

Sirodot, S. 1884. Les Batrachospermes, organisation fonctions, développement, classification. Libraire de Academie de Médicine, Paris, 299 pp.

Skinner, S. & Entwisle, T.J. (2001) Non-marine algae of Australia: 1. Survey of colonial gelatinous blue-green macroalgae (Cyanobacteria) Telopea 9: 573–599.

Skuja, H.1933. Untersuchengen uber die Rhodophyceen des Susswassers.III. *Batrachospermum* breutelii Rabenhorst und seine Brutkorper. Arch. Protistenk. 80: 350 – 366.

Skuja, H. 1934. Untersuchungen uber die Rhodophyceen des Susswassers.[IV-VI]. Beih. Bot. Centralb., Abt. B, 52: 173 – 192

Skuja, H. 1944: Untersuchungen Uberdie Rhodophyceen des Siisswassers. 8. *Nothocladus* lindaueri nov. sp. nebst einigen Beimerkungen fiber die Gattungen *Nothocladus* Skuja and *Tuomeya* Harvey. Ada Horti Botanici Universitatis, Latvia 14: 11-27, pi. 2-4.

Smith MA, Woodley NE, Janzen DH, Hallwachs W, Hebert PDN. 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proc Natl Acad Sci USA*, 103: 3657 – 62

Starmach, K.1977. Flora Slodkowodna Polski Tom 4: Phaeophyta – Brunatnice and Rhodophyta – Krasnorosty. Polska Academia Nauk, Warszawa, 425 pp.

Setchell, W. A. 1890. Contributions from the Cryptogamic Laboratory of Harvard University. XIII. Concerning the structure and development of *Tuomeya* fluviatilis, Harv. Proc. Am. Acad. Arts Sci. 25: 53– 68.

Stewart, S.A. & Vis, M.L. Investigation of two species complexes in *Batrachospermum* Section *Batrachospermum* (Batrachospermales, Rhodophyta). Phycologia (in revision Jan. 2007).

Stoeckle M. 2003. Taxonomy, DNA, and the Barcode of Life. *Bioscience*, 53: 796 – 797

Swale, E. M. F. & Belcher, J. H. 1963. Morphological observations on wild and cultured material of Rhodochorton investiens (Lenormand) nov. comb. (Balbiania investiens (Lenorm.) Sirodot). Ann. Bot. N.S. 27:281–90.

Swofford DL. 2000. PAUP*, Phylogenetic Analysis Using Parsimony (*and other methods), Version 4.0b10, Sinauer, Sunderland, Mass.

Tomas X. 1981. *Thorea ramosissima* en un canal del litoral Valenciano. *Fol Bot. Misc.*, 2: 71 – 74

Vis ML, Sheath, RG and Entwisle TJ.1995. Morphometric analysis of *Batrachospermum* section *Batrachospermum* (Batrachospermales, Rhodophyta) type specimens. Eur. J. Phycol. 31: 189 – 199

Vis, ML and Sheath RG.1996. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 9. Section *Batrachospermum*: desciption of five new species. Phycologia, 35: 124 – 134

Vis ML, Sheath, RG and Cole, KM. 1996a. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 8a. Section *Batrachospermum*: *Batrachospermum* gelatinosum. Eur. J. Phycol., 31: 31 – 40

Vis ML, Sheath, RG and Cole, KM. 1996b. Distribution and systematics of *Batrachospermum* (Batrachospermales, Rhodophyta) in North America. 8b. Section *Batrachospermum*: previously described species excluding *Batrachospermum* gelatinosum. Eur. J. Phycol., 31: 189 – 199

Vis ML, Sheath RG, Hambrook JA, and Cole KM.1994. Stream macroalgae of the Hawaiian Islands: a preliminary study. Pacific Science, 48: 175 – 187

Vis, M.L., Sheath, R.G. & Entwisle, T.J. (1995). Morphometric analysis of *Batrachospermum* section *Batrachospermum* (Batrachospermales, Rhodophyta) type specimens. Eur. J. Phycol., 30: 35 – 55.

Vis ML and Sheath RG. 1997. Biogeography of *Batrachospermum* gelatinosum (Batrachospermales, Rhodophyta) in North America based on molecular and morphological data. J. Phycol. 33: 520

Vis, ML and Sheath, RG.1998. A molecular investigation of the systematic relationship among Sorodotia species (Batrachospermales, Rhodophyta) in North America. J. Phycol., 34: (suppl.):61.(abstract)

Vis, M.L., Saunders, G.W., Sheath, R.G., Dunse, K. & Entwisle, T.J. (1998). Phylogeny of the Batrachospermales (Rhodophyta) inferred from *rbc*L and 18S ribosomal DNA gene sequences. J. Phycol., 34: 341–350.

Vis, M.L. & Entwisle, T.J. (2000). Insights into the phylogeny of the Batrachospermales (Rhodophyta) from *rbc*L sequence data of Australian taxa. J. Phycol., 36: 1175–1182.

Vis ML, Miller EJ and Hall MM.2001. Biogeographical analysis of *Batrachospermum* helminthosum (Batrachospermales, Rhodophyta) in North America using molecular and morphological data. Phycologia 40: 2 – 9

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN.2005. DNA barcoding Australia's fish species. *Philos Trans R Soc Lond B Biol Sci*, 360: 1847 – 1857

Webster, RN. The life history of the freshwater red algae *Tuomeya* fluviatilis Harvey. Butler Univ. Bot Stud. 13, 141 – 159

Wehr, J.D. & Sheath, R.G. 2003. Freshwater Habitats of Algae. pp. 11-57, In: Freshwater Algae of North America: Ecology and Classification. Academic Press, San Diego, CA.

Wetherbee R and Quirk HM.1982. The fine structure of secondary pit connection formation between the red algal alloparasite Holmsella australis and its red algal host Gracilaria furcellata. Protoplasma, 110: 167 - 176

Witt DSJ, Threloff DL, Hebert DN. 2006 . DNA barcoding reveals extraordinary cryptic diversity in an amphoid genus: implications for desert spring conservation. Molecular Ecology, 10:3073 – 3082

Woelkerling, W.J. 1990. An introduction. Pages 1-6 in Biology of the red algae (K.M. Cole and R.G. Sheath, eds.). Cambridge University Press, Cambridge.

Xie SL, Shi ZX and Wang RN. 2004. Taxonomy of the Lemaneaceae (Batrachospermales, Rhodophyta) in China. Acta Botanica Sinica 46 (8): 883 - 888

Yoon HS, Hackett JD, Pinto G and Bhattacharya D. 2002b. The single, ancient origin of chromist plastids. Proc. Natl. Acad. Sci. USA 99:15507–12.

Yoon HS, Müller KM, Sheath RG, Ott FD, Bhattacharya D.2006. Defining the major lineages of red alage (Rhodophyta). Journal of Phycology 42, 482-492

Yung Y.-K., Stokes P and Gorham E. 1986. Algae of selected continental and maritime bogs in North America. *Canadian Journal of Botany*, 64: 1825 – 1833