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New Collections of Freshwater Red Algae (Batrachospermales, Rhodophyta) from Historically Important Areas in France

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Abstract – An interest in freshwater red algae began in France over 200 years ago with naturalists, such as Bory de St. Vincent and Sirodot, who described numerous new taxa primarily within the Batrachospermales. Since the late 1800s, there has been limited research on these organisms in France. The present work was undertaken to revisit regions historically known to have a diversity of Batrachospermales. Overall, nine taxa were identified from 22 streams. Nine specimens representing six species were collected from eight streams near Rennes. From the Dax region, 16 specimens of six species were collected from 12 streams. Two streams were sampled near Vernon yielding two species. The *rbc*L gene was sequenced for all specimens and phylogenetically analyzed. Sequence data revealed that the Batrachospermales were broadly represented by the nine taxa collected. For most taxa, sequence data are available from other parts of the world, but these are the first for *Batrachospermum vogesiacum*. Although there have been changes in land use, such as road construction, many taxa documented by the pre 20th century French naturalists are still present in the flora today.

***Batrachospermum* / *Lemanea* / *Paralemanea* / *rbc*L gene / *Sheathia* / streams**

Résumé – **Collections modernes d'algues rouges d'eau douce (Batrachospermales, Rhodophyta) dans les régions historiques en France.** L'étude des algues rouges d'eau douce a été initiée, en France il y a deux siècles, par les naturalistes Bory de Saint-Vincent et Sirodot, qui ont décrit de nombreux nouveaux taxons principalement dans le Batrachospermales. Depuis lors, il y a eu peu de recherches sur ces organismes en France. La présente étude a été entreprise pour revisiter les sites dans lesquels les Batrachospermales avaient été historiquement reportés. Neuf taxons ont été identifiés dans 22 ruisseaux. Neuf spécimens représentant six espèces ont été recueillis dans huit ruisseaux près de Rennes. Dans la région de Dax, 16 spécimens de six espèces ont été recueillis dans 12 ruisseaux. Deux ruisseaux près de Vernon ont été échantillonnés ce qui a conduit à la récolte de deux espèces. Le gène *rbc*L a été séquencé pour tous les spécimens et une analyse phylogénétique a été faite. Pour la plupart des taxons, les données de séquence sont disponibles et proviennent d'autres régions du monde, cependant, nous reportons ici la première séquence pour *Batrachospermum vogesiacum*. Bien qu'il y ait eu des changements dans l'utilisation des terres et de l'habitat, de nombreux taxons étudiés par les naturalistes français avant le 20e siècle sont encore présents dans la flore d'aujourd'hui.

***Batrachospermum* / *Lemanea* / *Paralemanea* / gène *rbc*L / *Sheathia* / ruisseaux**

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INTRODUCTION

An interest in freshwater red algae began in France over 200 years ago (Entwisle, 1998). One of the first researchers to seriously study this group of organisms was Bory de St. Vincent in the late 1700s. As part of his interest as a naturalist, he described new species of both *Batrachospermum* and *Lemanea* from Thore's collections made near Dax in Gascony and from his own collections near Rennes in Brittany (Bory, 1797, 1808b, c). Thore was a medical doctor in Dax who collected red algae and corresponded with Bory de St. Vincent. Subsequently, Bory de St. Vincent named a new freshwater red algal genus, *Thorea*, in honor of Thore (Bory, 1808a).

In the mid to late 1800s, Sirodot and Gallée collected extensively in the vicinity of Rennes. These specimens formed the basis of Sirodot's seminal work on *Batrachospermum*, in which numerous new species were described (Sirodot, 1884). In addition to his studies of *Batrachospermum*, he also described species of *Lemanea* and *Paralemanea* from the Rennes area (Sirodot, 1872). Many of Sirodot's observations have guided taxonomic studies to date with numerous of the taxonomic entities he described still recognized today (Kumano, 2002).

Much of the freshwater red algal taxonomic diversity is in the order Batrachospermales (Kumano, 2002). This order includes *Batrachospermum, Sirodotia, Tuomeya, Lemanea* and *Paralemanea* (Kumano, 2002), and more recently *Kumanoa* (Entwisle *et al*., 2009) and *Sheathia* (Salomaki *et al*., 2014). The genus *Batrachospermum* has been known to be paraphyletic since the first molecular studies of the order and presently, there is work under way to describe new genera from the sections of *Batrachospermum* (Entwisle *et al*., 2009; Salomaki *et al*., 2014). Interestingly, many of the sections being raised to genera were those circumscribed by Sirodot. Previously, *Thorea* and the related genus *Nemalionopsis* were placed in the Batrachospermales, but after extensive morphological, anatomical and molecular analyses, a new order, the Thoreales, was erected for those genera (Müller *et al*., 2002).

Molecular systematic studies of the Batrachospermales have yielded many insights into the relationships of genera and species (Vis *et al*., 1998; Vis & Entwisle, 2000; Entwisle *et al*., 2009). Apart from the work done by Salomaki *et al*. (2014), the majority of specimens used in the molecular studies have been from North America, South America and Australasia. Only a handful of specimens have been sequenced from Europe and even fewer from France (Entwisle 1998). Since many species were first described from France, molecular data for specimens from this country would be keyed to providing insights about these taxa.

Since Sirodot's work, there has been limited research on these organisms in France. Hamel (1925) summarized the previous research and geographic distribution of species (Table 1). Since that time, only a few publications of freshwater red algae have occurred in broader studies or part of a larger work (*i.e.* Bourrelly, 1970) and there have not been any concentrated studies of these organisms in France. Entwisle (1998) documented a few collections made mostly near Rennes in 1992, but the primary focus of the research was a summary of the herbarium material at the Herbier Cryptogamique (PC). The purpose of the present research was to revisit streams and general regions that provided specimens for the studies in the 1700 and 1800s to search for freshwater red algae. In addition, if specimens were found, to morphologically identify them and provide sequence data in order to put them into a broader phylogenetic framework with specimens from other continents.

Table 1. Freshwater red algae from the order Batrachospermales previously reported from France. Records were compiled from Sirodot (1872, 1884), Hamel (1925) with nomenclature updated primarily from AlgaeBase (Guiry & Guiry, 2012) (accessed July 17, 2013)

Taxon	*Regions*
Batrachospermales	
Batrachospermum section *Batrachospermum*	
B. gelatinosum (Linnaeus) De Candolle (as *B. moniliforme* and many other synonymies – see Vis *et al*. 1995 for a complete list)	Brittany, Provence-Alpes-Côte d'Azur, Limousin, Lower Normandy, Pays-de-la-Loire, Île-de-France, Poitou-Charentes, Aquitaine, Languedoc-Roussillon, Burgundy, Lorraine, Poitou-Charentes,
B. skujae Geitler (as *B. sporulans* Sirodot)	Brittany
Batrachospermum section *Setacea*	
B. atrum (Hudson) Harvey (as *B. dilleni* Sirodot and *B. gallaei* Sirodot)	Brittany, Lower Normandy, Pays-de-la-Loire, Île-de-France, Centre, Aquitaine, Languedoc-Roussillon, Provence-Alpes-Côte d'Azur, Lorraine
Batrachospermum section *Turfosa*	
B. turfosum Bory [also as *B. vagum* (Roth) C. Agardh]*	Aquitaine, Brittany
B. keratophytum Bory [*B. vagum* var. *keratophytum* (Bory) Sirodot]	Aquitaine, Brittany
Batrachospermum section *Virescentia*	
B. elegans Sirodot[1] (also as *B. coerulescens* Sirodot)	Brittany, Lower Normandy, Midi-Pyrénées
B. helminthosum Bory (also as *B. bruziense* Sirodot, *B. testale* Sirodot, *B. viride* Sirodot[2], *B. virgatum* Sirodot)	Brittany
B. graibussoniense Sirodot[3]	Brittany
B. vogesiacum Schultz ex Skuja (as *B. vagum* var. *flagelliforme* Sirodot)	Brittany
Kumanoa	
K. virgatodecaisneana (Sirodot) Entwisle, M.L.Vis, W.B.Chiasson, Necchi & A.R.Sherwood	Brittany
Lemanea	
L. ciliata (Sirodot) De Toni	Brittany
L. fluviatilis (L.) C.Agardh	Brittany
L. fucina Bory	Brittany
L. mamillosa Kützing	Brittany
L. rigida (Sirodot) De Toni	Brittany
Paralemanea	
P. annulata (Kützing) M.L.Vis & R.G.Sheath	Brittany
P. catenata (Kützing) M.L.Vis & R.G.Sheath	Brittany
P. nodosa Kützing	Brittany
P. parvula (Sirodot) S.L.Xie & Z.X.Shi	Brittany
P. torulosa (Roth) R.G.Sheath & A.R.Sherwood	Brittany
Sheathia	
S. boryana (Sirodot) Salomaki & M.L.Vis (as *B. boryanum* Sirodot, *B. anatinum* Sirodot, *B. ectocarpum* Sirodot)	Brittany, Île-de-France, Aquitaine, Provence-Alpes-Côte d'Azur
S. confusa (Bory) Salomaki & M.L. Vis	Brittany, Lower Normandy, Aquitaine, Auvergne, Lorraine

*Distribution data from Hamel (1925) was not utilized because he grouped reports that may represent *B. turfosum, B. keratophytum* and *B. vogesiacum*.

[1]This is a currently recognized species according to AlgaeBase. However, there has been research that suggests it is may be a synonym of *B. helminthosum* (Vis *et al*., 2001). Since it has not been formally made a synonym, we continue to recognize it.

[2]*B. viride* was stated to be a synonym of *B. helminthosum*, but this species not being included in the taxonomic changes was probably an oversight (Sheath *et al*., 1994).

[3]Sheath *et al*. (1994) placed this taxon in synonymy with *B. helminthosum* based on Sirodot's (1884) description, but did not examine the type specimen of this taxon. Therefore, we have chosen to recognize it as a separate taxon until the type specimen can be examined and confirmed.

MATERIALS AND METHODS

Prior to field research, historical records for locations were gleaned from herbarium sheets housed at Muséum National d'Histoire Naturelle, Herbier Cryptogamique (PC) and Sirodot's monograph (Sirodot, 1884). As did Entwisle (1998), we attempted to pinpoint Sirodot's locations around Rennes, this time using Google Maps (2011). Many of the herbarium sheets noted "environs de Dax" with no specific site information. Therefore, Google Maps was consulted for potential sampling locations by examining photographs and streetview for roads crossing streams.

In the spring of 2008, four sites near Rennes were sampled. During fall 2011, 51 sites in the Rennes area were visited with four sites having freshwater red algae present. As well, 16 sites near Dax (Aquitaine), 12 with red algal taxa were sampled in fall 2011. A site near Vernon and one site near Giverny (Haute-Normandie) were sampled in fall 2013, respectively. In total, red algae were collected from 22 sites (Fig. 1, Table 2).

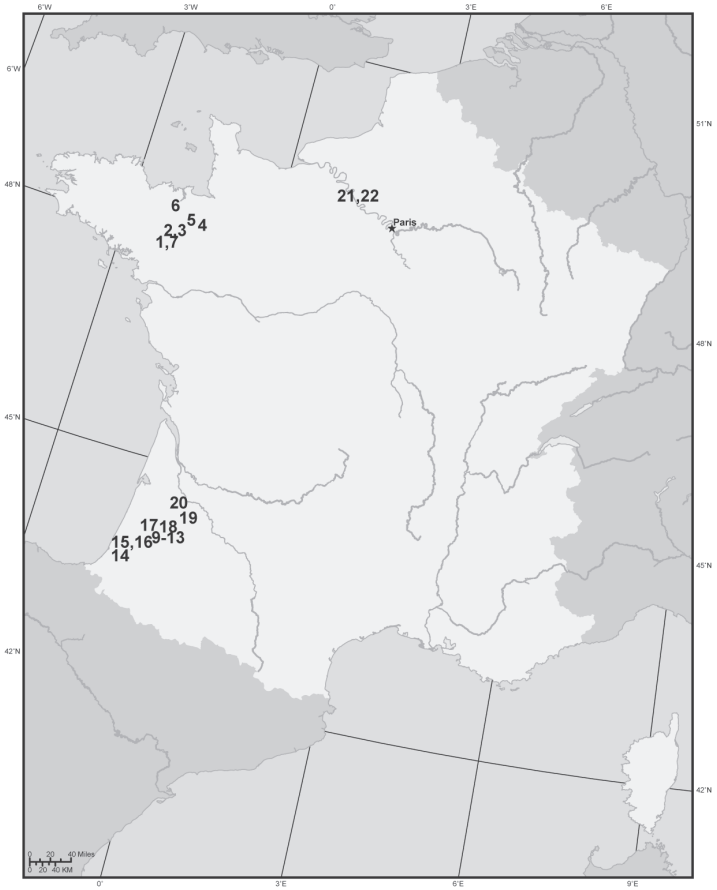

Fig. 1. Map of France showing the 22 sampling localities near Rennes, Dax and Vernon.

Table 2. Collection information and stream characteristics for locations, from which freshwater red alga taxa were sampled. Genbank accession numbers for the *rbc*L gene sequence data from specimens of *Batrachospermum, Lemanea*, *Paralemanea* and *Sheathia*

Stream Number	*Collection Information (Location, latitude, longitude, collectors, date, herbaria with voucher)*	*pH*	*Specific Conductance ($\mu S.cm^{-1}$)*	*Water temp. (°C)*	*Canopy Cover (%) Water Depth (cm)*	*Taxon*	*rbcL GenBank Number*
1	La Fontaine de Barenton, spring outlet in manmade enclosure, in Forêt Paimpont, 48.03885, –2.246717, M. Vis & W. Chiasson 25.iii.2008, BHO A-0132, MICH.	5.1	120	11	0 –	*B. turfosum*	KJ825960
2	Boucle de l'Enchateur, ruisseau de Pont Dom Jean, Forêt Paimpont, 48.075597, –2.009633, M. Vis & W. Chiasson, 26.iii.2008, BHO A-0082, MICH.	6.9	130	10	80 45	*B. gelatinosum*	GU810834
3	Vallon de la chambre au loup, Forêt Communale d'Iffendic, 48.09485, –2.057233, M. Vis & W. Chiasson 26.iii.2008, BHO A-0084, A-0237, MICH.	7.2	150	9	50 40	*B. gelatinosum* *L. fucina*	KJ825964 KJ825958
4	Vallée du Couesnon, small stream flowing into the Couesnon River, 48.31195, –1.431217, M. Vis & W. Chiasson, 27.iii.2008, BHO A-0239.	7.5	240	8	40 40	*L. fucina*	KJ825959
5	Le Boulet Prioult, near Le Petit Boulet, 48.332722, –1.666667, W. Chiasson & E. Salomaki, 5.x.2011, BHO A-0901.	7.2	210	19	80-90 12	*B. helminthosum*	KJ825955
6	Chateau de Montefilan ruins parking lot. Corseul, France. 48.487444, –2.190694, W. Chiasson & E. Salomaki, 6.x.2011, BHO A-0905 ISOTYPE, MICH.	7.4	530	15	0 > 100	*S. exigua*	JX669738
7	On walking trail at Paimpont Abbey, spring outlet in manmade enclosure, 48.0235, –2.179389, W. Chiasson & E. Salomaki, 7.x.2011, BHO A-0907.	6.1	90	12	60 –	*B. turfosum*	KJ825961
8	Site along road between Iffendic and Montfort-sur-Meu, 48.136111, –2.001306, W. Chiasson & E. Salomaki, 8.x.2011, BHO A-0910.	–	510	–	75 –	*P. catenata*	KJ825957
9	Stream crossing Rt 14 towards Villenave, 43.944583, –0.802361, M. Vis, W. Chiasson & K. Chiasson 26.x.2011, BHO A-0922.	7.8	210	11	– 40	*B. gelatinosum*	KJ825965
10	Just north of Villenave, a washing trough with cement sides, 43.970528, –0.806306, M. Vis, W. Chiasson & K. Chiasson 26.x.2011, BHO A-0923.	6.5	170	11	– > 1 m	*B. gelatinosum*	KJ825966
11	Fontaine de Cla, the springhead in a cement enclosure, 44.005167, –0.733722, M. Vis, W. Chiasson & K. Chiasson 26.x.2011, BHO A-0924.	5.4	120	15	– –	*B. turfosum*	KJ825962

Table 2. Collection information and stream characteristics for locations, from which freshwater red alga taxa were sampled. Genbank accession numbers for the *rbc*L gene sequence data from specimens of *Batrachospermum, Lemanea*, *Paralemanea* and *Sheathia (continued)*

Stream Number	*Collection Information (Location, latitude, longitude, collectors, date, herbaria with voucher)*	*pH*	*Specific Conductance ($\mu S.cm^{-1}$)*	*Water temp. (°C)*	*Canopy Cover (%) Water Depth (cm)*	*Taxon*	*rbcL GenBank Number*
12	At spring outflow, St Jean, 43.947667, –0.724, M. Vis, W. Chiasson & K. Chiasson 26.x.2011, BHO A-0925.	6.0	190	14	– 15	*B. gelatinosum*	KJ825967
13	River at St Jacques, 43.929083, –0.731194, M. Vis, W. Chiasson & K. Chiasson 26.x.2011, BHO A-0926, A-0927.	6.9	210	14		*B. vogesiacum,* *S. confusa*	KJ825953 KJ825971
14	Ruisseau Jouanin at Saubusse, 43.659917, –1.181806, M. Vis, W. Chiasson & K. Chiasson 27.x.2011, BHO A-0928.	7.3	240	14	– –	*B. gelatinosum*	KJ825968
15	Stream crossing D142, 43.877944, –1.228611, M. Vis, W. Chiasson & K. Chiasson 27.x.2011, BHO A-0929.	7.0	120	14	– 30-40	*S. confusa*	KJ825972
16	La Paulie, Rt. 374 north of Commune St. Michel Escalus, 43.88075, –1.248861, M. Vis, W. Chiasson & K. Chiasson 27.x.2011, BHO A-0930, A-0931.	7.0	120	14	– –	*B. vogesiacum* *S. confusa*	KJ825954 JX669739
17	La Grande Leyre, crossing D44, 44.152111, –0.790833, M. Vis, W. Chiasson & K. Chiasson 27.x.2011, BHO A-0932.	6.5	130	14	– 30	*S. confusa*	KJ825973
18	Steam near D626, 44.11025, –0.580194, M. Vis, W. Chiasson & K. Chiasson 29.x.2011, BHO A-0933, A0934.	6.5	110	14	– 30	*B. turfosum* *B. gelatinosum*	KJ825963 KJ825969
19	Dirt road to Chappelle Saint Clair-de-Gout, off of D433, 44.237472, –0.058806, M. Vis, W. Chiasson & K. Chiasson 29.x.2011, BHO A-0935, A-0936.	7.2	120	15	– –	*B. helminthosum* *S. confusa*	KJ825956 JX669740
20	Small stream on the grounds of Château de Cazeneuve, 44.38778, –0.319028, M. Vis, W. Chiasson & K. Chiasson 29.x.2011, BHO A-0937.	7.5	250	16	– –	*S. exigua*	KJ825974
21	Château de Saint, in a pond behind the chateau, into which a spring flowed, Vernon, Normandy. 49.10995, 1.43173, M. Vis & B. de Riviers, 13.x.2011, BHO A-0911.	8.0	640	15	– –	*S. arcuata* (*Hildenbrandia* sp.)	JX669741
22	Giverny, Normandy. 49.07454, 1.53517, B. de Riviers, 28.iv.2013, BHO A-1107.	–	–	–	– –	*B. gelatinosum*	KJ825970

A wide range of freshwater sites was visited including small springs, lavoires, wadeable, and non-wadeable streams (Figs 2-5). At each site, a minimum of 20 m was searched, when possible, as some sites were small springs with a much smaller area of water. For most sites, the pH, specific conductance and temperature were measured with handheld probes. Canopy cover and mean water depth were estimated (Table 2). The latitude and longitude were recorded from a GPS unit. Samples of each taxon encountered were collected with a portion preserved in silica gel for DNA and the other pressed on herbarium paper as a morphological voucher and deposited in the Bartley Herbarium Ohio University (BHO).

Figs 2-5. Representative stream habitats sampled. Stream numbers as in Table 2. **2.** Small man made spring enclosure, site 12. **3.** A lavoire, in which the algae is attached to the concrete sides, site 5. **4.** A typical stream, ~ 7 m wide with overhanging trees, site 8. **5.** Larger river, ~ 25 m wide with large boulders and aquatic plants, site 16.

Samples for DNA analyses were ground by hand in liquid nitrogen using a mortar and pestle. DNA was extracted with the NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany) kit according to the manufacturer's protocol. The *rbc*L gene was PCR amplified using either a MJ Research Minicycler™ (Bio-Rad, Hercules, CA, USA) or the Applied Biosystems 2720 Thermocycler™ version 2.08 (Applied Biosystems, Foster City, CA, USA). A 1,282 bp fragment the *rbc*L gene was amplified using the F160 and *rbc*LR primers (Vis *et al*., 1998). The PCR cocktail consisted of 19 µL dH_20, 25 µL AmpliTaqGold master mix (Applied Biosystems, Carlsbad, CA, USA), 2.5 µL each of the amplification primers and 1 µL extracted DNA. The PCR parameters were as follows: an initial denaturing at 95°C for 1:00; 35 repeated cycles of 93°C for 0:30, 50°C for 0:30 and 68°C for 1:00; a final elongation period at 72°C for 10:00. PCR products were purified using the UltraClean' PCR Clean-up DNA purification kit (Mo Bio, Carlsbad, CA, USA) according to manufacturer's protocols.

The purified PCR products were sequenced using the PCR amplification primers. The additional internal primers F650 (5'-ATT AAC TCT CAA CCA TTT ATG CG-3'), R897.1 (5'-CGT GAG TAT GTT GAA TTA CCA GC-3'), R897.3 (5'-CGT GAA TAT GTA GAG TTA CCT GC-3') and R897.test (5'-CGT GAG TAT GTT GAA TTA CCT G-3') were used to ensure that the 1282 bp fragment was fully sequenced in both directions. All DNA sequences were assembled and edited using Sequencher' version 4.10.1 (GeneCodes Corp, Ann Arbor, MI, USA). Newly generated sequence data from this study were uploaded to GenBank (Table 2).

To place these collections in context with known diversity, sequence data from specimens collected during this survey were combined with 60 sequences for all other Batrachospermales genera downloaded from GenBank (accessed June 16, 2013) (Table 3). The dataset was outgroup rooted with members of three closely related orders, *Audouinella arcuata* (Drew) Garbary, G. I. Hansen & Scagel, *Ballia callitricha* (C. Agardh) Kütz., and *Thorea violacea* Bory. Sequence alignment was completed using Geneious Pro 6.0.5 (Biomatters Ltd., Auckland, New Zealand), and the best model for evolution was determined using the BIC as implemented in jModelTest v0.1.1 (Posada, 2008). For this dataset, the model was: TIM3 substitution model with a gamma distribution = 0.8270; proportion of invariable sites = 0.5260; base frequencies A = 0.3941, C = 0.1036, G = 0.1206, T = 0.3817; and rate matrix A-C = 3.7809, A-G = 7.9878, A-T = 1.0000, C-G = 3.7809, C-T = 22.0453, and G-T = 1.0000. The dataset was subjected to Bayesian Inference (BI) analysis using MrBayes v3.2 (Ronquist *et al*., 2012) and maximum likelihood (ML) analysis using RAxML (Stamatakis, 2006). For the BI analyses, two Metropolis-coupled Markov chain Monte Carlo (MCMCMC) runs consisting of one cold chain and three hot chains were preformed. Each run was sampled every 100 generations for 5,050,000 generations. After confirming that the runs converged by checking to ensure that the average standard deviation of split frequencies was below 0.01, the trees were merged following the removal the first 500 trees from each run as burn-in. The resulting tree and posterior probabilities were calculated from the remaining 100,000 trees generated for all datasets. The model parameters for the ML analyses were the same as those for the BI. ML bootstrap support values were calculated using 1,000 bootstrap replicates. The alignment of *rbc*L sequences used for phylogenetic analyses in this study is available at http://purl.org/phylo/treebase/phylows/study/TB2:S15799.

Table 3. Sequence accession numbers for taxa used in phylogenetic analyses of the order Batrachospermales

Taxon	*GenBank Accession Number*
Audouinella arcuata	AF029138
Ballia callitricha	AF149029
Balliopsis prieurii	AY960688
Batrachospermum antipodites	AY423421
B. atrum	AF029139
B. brasiliense	FJ386458
B. cayennense	AF209980, AY423392
B. gelatinosum	AF029141, EF375888, KJ825965
B. helminthosum	AB114642, AB114643, AB114644, AB114645, AB114646, AF244109, AF244115, AF244116, AF244117, AF029142, KJ825955
B. macrosporum	AY423417, EU106049
B. pseudogelatinosum	AF209983
B. turfosum	AF029147, AY423407, DQ449028, KJ825961, KJ825963
B. vogesiacum	KJ825954
Kumanoa americana	JN589995
K. tabagatenensis	JN590009
Lemanea borealis	AF029149
L. fluviatilis	AF029150 AY575149 AY575157 AY575164 AY575168
L. fucina	AY575158, AY575166, KJ825958
L. fucina var. *parva*	AF029151
Nothocladus nodosus	AF029152
Paralemanea annulata	DQ449029 GQ285124 U04038
P. catenata	AF029154, JF701686, KJ825957
P. grandis	DQ523258, DQ523259
Paralemanea sp.	JF701688
Petrohua bernabei	AY960690
Psilosiphon scoparum	AF029155
Sheathia americana	AF029140
S. arcuata	JX669741, DQ393129, DQ393131 GU457346, EF116873
S. boryana	JX669773
S. confusa	JX669739, JX669740
S. exigua	GU457344, JX669738, KJ825974
S. grandis	JX669803
S. involuta	AF029143
S. heterocortica	DQ393136
Sirodotia delicatula	DQ646475
S. huillensis	AF029157
S. suecica	JF344718
Thorea violacea	AF029160
Tuomeya americana	AF029159

RESULTS

The 22 streams sampled with freshwater red algal taxa varied in size and stream parameters (Figs 1-5, Table 2). Among the eight sites near Rennes, the pH varied from 5.1 to 7.5, specific conductance ranged from 90-530 $\mu S \cdot cm^{-1}$ and estimated canopy cover was open to highly shaded. The temperature varied, but sites were sampled in two different seasons (Table 2). The 12 sites near Dax showed variation in pH (5.4-7.8), specific conductance (110-250 $\mu S \cdot cm^{-1}$), but all sites had similar water temperature (11-16°C). The one Vernon site had basic pH (8.0) and high specific conductance (640 $\mu S \cdot cm^{-1}$). Most of the locations near Rennes had rocky bottoms and the algae were on those rocks or were springs with man-made enclosures. At many of streams in the Dax area, the stream bottom was dominated by sand and the algae were clinging to larger rocks and other stable substrates (i.e. large logs, man-made structures). Likewise, at site 21 near Vernon there were few rocks and the algae was collected from those.

Twenty-seven specimens were collected from the 22 sites (Table 2). At 17 sites, a single species was collected and at five locations there were two species. *Batrachospermum gelatinosum* was the most abundant species having been collected in eight streams and present in all three areas sampled, Rennes, Dax and Vernon. *Batrachospermum turfosum*, *B. helminthosum* and *Sheathia exigua* were all present in the Rennes and Dax regions. The species, *B. turfosum* was in two streams in each region. *Sheathia confusa* was only found in the Dax region, but was present in five of the 12 streams. The remaining four taxa (*Lemanea fucina, Paralemanea catenata, Batrachospermum vogesiacum* and *Sheathia arcuata*) were collected in a single region. Seven of the species were sampled from more than one stream and only *Paralemanea catenata* and *Sheathia arcuata* were only collected in a single stream (Table 2).

Sequence data from the 1282 bp portion of the *rbc*L gene was generated for all nine taxa collected. For the seven species with more than one specimen, there was high within species similarity. The seven specimens of *B. gelatinosum* were identical to each other. Likewise, four of the *S. confusa* specimens had identical sequence to each other with the fifth specimen 1 bp different. Of the three *B. turfosum* specimens, two were identical and the third was 1 bp different. The two *Sheathia exigua* specimens differed by 1 bp. The two specimens of *B. helminthosum* were identical as were the two specimens of *B. vogesiacum*. For all species, except *B. vogesicacum*, there were sequence data available from other parts of the world. When the data from the new specimens were compared with those, most were quite similar (≤ 1% sequence variation in a BLAST search on GenBank). The only two exceptions were *B. turfosum* differing from previously reported sequences by 31-34 bp (2.4-2.7%) and *Paralemanea catenata* differing by 49 bp (3.8%).

Phylogenetic analyses using Bayesian Inference (BI) and Maximum Likelihood (ML) showed similar topologies such that only the ML is shown (Fig. 6). The nine species collected were distributed throughout the Batrachospermales. *Batrachospermum gelatinosum* was in a well-supported clade with specimens of that taxon from the UK and North America. The *Paralemanea catenata* specimen was within the well-supported *Paralemanea* clade, but was not closely allied with any other specimens, even those identified as *P. catenata*. The *Lemanea fucina* specimen was closely related to specimens from the UK and Sweden, some of which were identified as *L. fluviatilis*. The specimens of *Sheathia confusa* were in a well-supported clade with *S. americana*. The two *S. exigua* specimens were in a

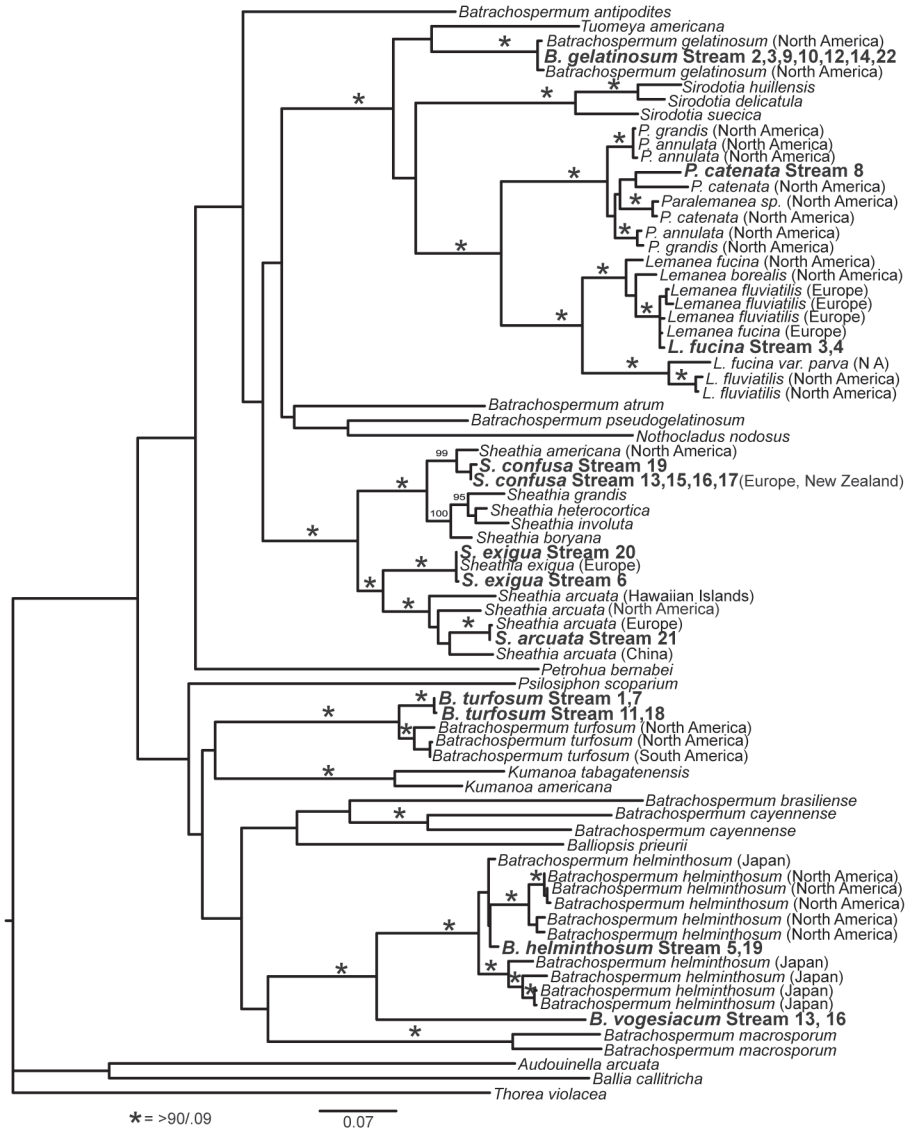

Fig. 6. Phylogenetic tree derived from ML analysis (log likelihood = –15977.092440) of *rbc*L data for specimens from this study and previously published data. Only branches with > 90% ML bootstrap and 0.90 posterior probability are marked. Stream numbers as in Table 2.

well-supported clade with another specimen of that taxon from Bulgaria. Likewise, *S. arcuata* was within a well-supported clade of specimens from that taxon. The *B. turfosum* specimens from France were sister to specimens of *B. turfosum* from North and South America. Both of these clades were well supported. *Batrachospermum helminthosum* was within a well-supported clade of like named specimens and *B. vogesiacum* was sister to the *B. helminthosum* clade (Fig. 6).

DISCUSSION

This survey has provided insights into taxonomic relationships within the Batrachospermales, but also has highlighted the need for more taxonomic work. *Batrachospermum gelatinosum* is widely distributed in both North America and Europe, but differs by only a few base pairs in the *rbc*L gene. Likewise, *Sheathia confusa* has been recorded from very distant locations in New Zealand and Europe, but with little sequence variation. *Sheathia exigua* also had little sequence variation, but may be restricted to Europe. *Batrachospermum vogesiacum* from the section *Virescentia* was confirmed as distinct from *B. helminthosum* in both morphology and molecular data. However, it is clear that *B. helminthosum* is genetically diverse and may represent more than one taxon. Likewise, specimens attributed to *Sheathia arcuata* may harbor cryptic species. Specimens identified as *Paralemanea catenata* and *Batrachospermum turfosum* based on morphology had quite divergent sequence data from specimens attributed to those species from other continents. As well, the specimens of *Lemanea fucina* were more closely related to specimens identified as *L. fluviatilis* than some of the other *Lemanea fucina* specimens. These sequence data of specimens from France will be important in further taxonomic revisions. These molecular data hint that specimens attributed to species first described from Europe, may be new species and that the red algal flora of Europe may not be as similar to North America as previously presumed.

The present survey was limited in scope, both geographically and number of streams sampled. Nevertheless, 27 specimens of nine species in the Batrachospermales were collected. Historical records denoted 12 species belonging to *Batrachospermum* (including the two newly described genera *Kumanoa* and *Sheathia*) and this survey collected five of those (*Batrachospermum gelatinosum, B. helminthosum, B. turfosum, B. vogesiacum,* and *Sheathia confusa*) (Tables 1, 2). In addition to these five, two new taxa were added to the flora, *S. arcuata* and *S. exigua* (type location site 6). There were seven previously reported taxa, but not surveyed as follows: *Batrachospermum skujae, B. atrum, B. keratophytum, B. elegans, B. graibussoniense, Sheathia boryana* and *Kumanoa virgatodecaisneana*. *Batrachospermum skujae* is morphologically similar to *B. gelatinosum*, but has monospores (Kumano, 2002). This taxon has only been reported from a handful of locations in Europe and North America such that modern collections and sequence data are needed to determine its taxonomic validity (Vis *et al*., 1995; Eloranta *et al*., 2011). *Batrachospermum atrum* has been reported throughout Europe including the UK and its absence in the survey was unexpected. The type localities for both *B. keratophytum* and *B. turfosum* are in the Dax region. However, only one genetic entity was collected in the survey. The species, *B. elegans* is in section *Virescentia* and closely related to *B. helminthosum*. It has been suggested that the distinguishing feature of *B. elegans*, knobs on the trichogyne, is environmentally induced and may be a variant of *B. helminthosum* (Vis *et al*., 2001). In describing *B. graibussoniense*, Sirodot (1884) noted that this taxon was from a single stream. The exact location at which Sirodot sampled could not be ascertained, but a few streams in the general region were surveyed with no freshwater reds found. From other research, *S. boryana* is known to be widespread and abundant in Europe so its absence from the survey was surprising (Salomaki *et al*., 2014). However, *S. exigua*, which is somewhat morphologically similar, was found in the general region. In the present survey, *Kumanoa virgatodecaisneana* was not collected, but it has been reported in recent times near

Rennes (Entwisle, 1998). This location was visited in the present survey, but no algae were found, potentially due to new road construction or drought conditions.

Brittany, in particular Rennes, was a focus of the survey since this was the area sampled by Sirodot for his 1884 monograph. Additionally, Bory de St. Vincent collected in this region. Rennes was visited in the spring 2008 and only a few streams could be sampled due to heavy rains. Conversely, there were drought conditions when this area was visited in autumn 2011. The heavy rain and drought conditions when this area was sampled, could easily lead to an underestimation of the number of streams with freshwater reds and the richness of the area. There undoubtedly have been land use changes in the intervening 200 years since Sirodot and Bory de St. Vincent collected. However, it is difficult to draw conclusions regarding the effect of land use change due to the poor weather conditions (especially the extended drought) for the present day sampling. Nevertheless, this survey collected six batrachospermalean taxa from the region. As well, Entwisle (1998) reported *B. helminthosum*, *S. confusa* (as *B. confusum*, two locations) and *K. virgatodecaisneana* (as *B. virgatodecaisneanum*) from a small survey of the area around Rennes in 1992. Combining these more recent surveys, the total collected in the Rennes area is eight taxa.

In addition to the Rennes area, the region around Dax was explored since there had been numerous collections from the 1700s. In this region there were springs, lavoires and streams to sample. Many of the streams had sandy bottoms with a few rocks, which was a contrast to the rocky bottom streams of the Rennes area. The Dax region was visited in autumn 2011 and in contrast to Rennes, the stream conditions seemed favorable for sampling freshwater reds. With these non-drought conditions, 12 of the 16 streams sampled had freshwater red algae. Sixteen specimens of six taxa were collected in all. The present survey recollected *B. gelatinosum, B. turfosum* and *S. confusa* previously known from the region, but did not find three (*B. atrum*, *B. keratophytum* and *S. boryana*) previously reported for the region. However, *B. helminthosum*, *B. vogesiacum* and *S. exigua* were recorded for the first time from the region.

From this survey, it is obvious that there is the potential for numerous collections of freshwater red algal diversity in France. There are many diverse stream habitats that were sampled in the survey and others, such as montane regions, have yet to be explored. Recently, a colleague sent a specimen of *Paralemanea* from near Auriac in the Limousin region. Although not the focus of this survey for Batrachospermales, *Hildenbrandia*, a crustose freshwater red in the Hildenbrandiales, was present in one of the sites collected and potentially may be found in other regions. *Thorea* (Thoreales) was not collected in the present study, but this genus tends to be in larger rivers such as the Seine rather than the wadable rivers surveyed. As well, the macroscopic gametophyte of *Thorea* is only seasonally present, usually in fall such that it would not be evident in a spring sampling. Clearly, expanded collecting in other regions and seasons would yield more freshwater red algal reports and probably species diversity.

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