Cryptogamie, Algologie, 2010, 31 (4): 403-421

Establishment of a DNA-barcode library for the Nemaliales (Rhodophyta) from Canada and France uncovers overlooked diversity in the species *Nemalion helminthoides* (Velley) Batters

Line LE GALL [a*] *& Gary W. SAUNDERS* [b]

[a] *Muséum National d'Histoire Naturelle (MNHN)*
UMR 7138 Systématique, adaptation, évolution
Case postale N° 39, 57 rue Cuvier, 75231 cedex 05 Paris, France

[b] *Centre for Environmental & Molecular Algal Research*
Dept of Biology, University of New Brunswick
Fredericton, NB, E3B 5A3, CANADA

Abstract – Previous studies have established that the DNA barcode is useful to delineate species boundaries among red algae and have demonstrated that our understanding of red algal biodiversity and biogeography is fragmentary. In this context, we are establishing DNA-barcode libraries for red algae of the Canadian and French floras. In the present study we provide results regarding diversity for members of the red algal order Nemaliales. We have analyzed 87 individuals from the coasts of Canada and France, which spans three oceans (Pacific, Arctic, Atlantic) and two seas (English Channel and Mediterranean Sea). The 15 expected species of Nemaliales were all recovered with infraspecific distances ≤ 1% with the exception of *Nemalion helminthoides* (Velley) Batters, which was resolved as five divergent (10 to 18%) lineages. Two specimens from Brittany and Asturias were assigned to *Nemalion helminthoides*, collections from the Pacific were tentatively assigned to *Nemalion vermiculare* Suringar, and we resurrected *Nemalion lubricum* Duby for the Mediterranean specimen and *Nemalion multifidum* (F. Weber & D. Mohr) Chauvin for specimens collected on both sides of the northern Atlantic Ocean. We also uncovered the crustose phase of *Scinaia interrupta* (A.P. de Candolle) M.J. Wynne in Pacific Canadian waters, which represents a significant extension in the known range for this species. Finally, the placement of *Whidbeyella cartilaginea* Setchell & N.L.Gardner within the Scinaiaceae, a decision based tentatively on morpho/anatomical attributes, was confirmed by Bayesian analyses of large subunit ribosomal DNA (LSU).

DNA-barcoding / Nemaliales / *Nemalion helminthoides* / *Nemalion lubricum* / *Nemalion multifidum* / *Nemalion vermiculare*

Résumé – De nombreuses études ont montré l'utilité du code à barres ADN pour définir les frontières des espèces d'algues rouges et ont démontré en outre que notre compréhension de la diversité biologique et de la biogéographie des représentants de ce groupe était loin d'être complète. Nous avons donc constitué des bibliothèques de séquences code-barres ADN des algues rouges canadiennes et françaises. Dans le présent article, nous présentons des résultats concernant la diversité de représentants des Nemaliales. Quatre-vingt-sept individus des côtes du Canada et de la France ont été

* Correspondance and reprints: legall@mnhn.fr

étudiés, avec un échantillonnage incluant des spécimens provenant de trois océans (Pacifique, Arctique, Atlantique) et deux mers (la Manche et la Méditerranée). Nos séquences confirment bien la présence et la définition de chacune des 15 espèces de Nemaliales que nous nous attendions à récolter dans ces régions, les séquences de leurs représentants ayant entre elles des divergences génétiques ≤ 1 %, à l'exception de *Nemalion helminthoides* (Velley) Batters dont il est apparu que les représentants étudiés constituent cinq lignées différentes, distantes génétiquement entre-elles de 10 à 18 %. Deux spécimens de Bretagne et des Asturies ont été rapportés à *Nemalion helminthoides* ; nous avons proposé provisoirement de nommer nos récoltes du Pacifique *Nemalion vermiculare* Suringar mais une séquence d'un spécimen de la localité type est nécessaire pour avoir une certitude ; nous avons ressuscité le nom *Nemalion lubricum* Duby pour le spécimen méditerranéen de ce genre et ressuscité le nom *Nemalion multifidum* (F. Weber *et* D. Mohr) Chauvin pour des spécimens récoltés des deux côtés de l'océan Atlantique nord et différant morphologiquement de *N. helminthoides*. Nous avons aussi découvert le stade encroûtant de *Scinaia interrupta* (A.P. de Candolle) M.J. Wynne dans les eaux pacifiques canadiennes, ce qui représente une extension significative de la zone de distribution actuellement connue de cette espèce. Enfin, nos analyses Bayésiennes du gène nucléaire codant pour l'ARN ribosomique 28S (*large subunit ribosomal DNA* ou LSU) confirment que *Whidbeyella cartilaginea* Setchell *et* N.L. Gardner doit être placé dans les Scinaiaceae ; cette position systématique avait jusqu'ici été proposée sans certitude, sur la base de l'existence, chez cette espèce, de caractéristiques morpho/anatomiques particulières.

Code-barre ADN / Nemaliales / *Nemalion helminthoides* / *Nemalion lubricum* / *Nemalion multifidum* / *Nemalion vermiculare*

INTRODUCTION

In the current taxonomic framework, the Nemaliales is the representative order of the Nemaliophycidae, one of the five florideophycean subclasses (Saunders & Hommersand, 2004; Le Gall & Saunders, 2007). Within the Nemaliophycidae, the Nemaliales has strong affinities with the Acrochaetiales, Colaconematales and Palmariales (Harper, 2002; Le Gall & Saunders, 2007). The taxonomic history of the Nemaliales has been convoluted and we discuss only the major changes that have occurred within this order; the reader is referred to Huisman (2004a, 2006) for a comprehensive account. The Nemaliales was established by Schmitz (1892) to accommodate floridean taxa with simple postfertilization development in which the gonimoblast developed directly from the zygote (postfertilization carpogonium) in the absence of an auxiliary cell. Later Fristch (1945) redefined the limits of the order by including nine families (Acrochaetiaceae, Batrachospermaceae, Bonnemaisoniaceae, Chaetangiaceae, Helminthocladiaceae, Lemaneaceae, Naccariaceae, and Thoreaceae). The Acrochaetiaceae, Batrachospermaceae, Bonnemaisoniaceae, and Thoreaceae are now generally accorded ordinal status as Acrochaetiales (Feldmann, 1953), Batrachospermales (Pueschel, 1982), Bonnemaisoniales (Feldmann & Feldmann, 1942), and Thoreales (Müller, 2002), respectively. The Lemaneaceae was included in the order Batrachospermales (Pueschel, 1982) while the taxonomic affinities of the Naccariaceae are yet to be resolved (Schils, 2003). The successive removal of families from the Nemaliales since Fristch (1945) left the order with only the Chaetangiaceae and Helminthocladiaceae. In 1983, Parkinson replaced the name Chaetangiaceae with Galaxauraceae to resolve nomenclatural problems connected to the name *Chaetangium*. The Helminthocladiaceae, described by J. Agardh (1851), was replaced

by the earlier Liagoraceae (Kützing, 1843) despite a proposal to conserve the former (see Silva *et al.*, 1996, p. 119).

Huisman *et al.* (2004a) provided the first comprehensive molecular phylogeny for the Nemaliales using large-subunit ribosomal DNA sequences (LSU). They confirmed monophyly for the order, but challenged its infraordinal classification. Based on their molecular results they considered the Nemaliales to consist of three major lineages: one consisting of a restricted Galaxauraceae; a second lineage consisting of the Liagoraceae; and finally the new family Scinaiaceae for *Scinaia* and *Nothogenia*, and tentatively the genera *Gloiophloea* and *Whidbeyella*.

In the Canadian flora, *Cumagloia andersonii* (Farlow) Setchell *et* N.L. Gardner and *Nemalion helminthoides* (Velley) Batters, Liagoraceae, and *Scinaia confusa* (Setchell) Huisman, Scinaiaceae, are the only common representatives of this order. The rather enigmatic *Whidbeyella cartilaginea,* a putative member of the Scinaiaceae, has been reported from Union Island, British Columbia (Garbary, 1982). *Cumagloia andersonii* and *Scinaia confusa* are also restricted to the west coast (Gabrielson *et al.*, 2006), whereas *Nemalion helminthoides* occurs in both the Atlantic and Pacific (Sears, 2002; Gabrielson *et al.*, 2006). The Nemaliales of the French flora were thoroughly detailed by Hamel (1925). He recognized one species of Galaxauraceae, *Tricleocarpa fragilis* (Linnaeus) Huisman *et* R.A.Townsend (as *Galaxaura oblongata* (J. Ellis & Solander) J.V. Lamouroux*)*, two species of *Scinaia* (Scinaiaceae), *S. furcellata* (Turner) J. Agardh and *S. interrupta* (A.P. de Candolle) M.J. Wynne (as *S. subcostata* (J. Agardh) Chemin *ex* G. Hamel), two species of *Liagora*, *L. distenta* (Mertens *ex* Roth) J.V. Lamouroux and *L. viscida* (Forsskål) C. Agardh, and two species of *Nemalion*, *N. helminthoides*, mostly unbranched and occurring along both the Mediterranean and Atlantic shores, and *N. multifidum*, strongly branched and restricted to the Atlantic coast. However, Feldman (1954) considered *Nemalion multifidum* as a synonym of *N. helminthoides* recognizing only a single species of this genus in France. In fact, the later taxon reportedly has a wide distribution recorded from New Zealand (e.g. Levring, 1956), Australia (e.g. Womersley, 1965), Europe (Atlantic (e.g. Feldmann, 1954), North Sea, and Mediterranean (e.g. Hamel, 1925)), as well as the west and east coasts of North America (e.g. Gabrielson *et al.*, 2006; Sears, 2002).

In this study we establish a DNA barcode library for the Nemaliales of the Canadian and French coasts, which spans three oceans (Arctic, Atlantic, Pacific) and two seas (English Channel and Mediterranean), emphasizing the supposedly cosmopolitan *Nemalion helminthoides*.

MATERIAL AND METHODS

Sample collection, morphological and anatomical analyses

Specimens (Table 1) were collected in the intertidal or by scuba and preserved by mounting on herbarium paper with a subsample placed in silica gel for subsequent molecular analyses. Tissue for anatomical study was excised from the specimens, rehydrated in a 10% detergent (in freshwater) solution for one to three minutes, and then fixed with 4% formaldehyde (in freshwater) for 30 minutes. Sections were obtained by hand or with the aid of a freezing microtome Leica CM1850. Section thickness was typically between 45-60 µm for

Table 1. List of samples used in this study with collection details and BOLD accession numbers

Taxa	*Voucher*	*Collection details*	*Collectors*	*BOLD Accession numbers*
Palmariaceae (outgroup)				
Palmaria palmata (Linnaeus) Weber *et* Mohr	LLG1775	Saint Lunaire, Ille-et-Vilaine, Brittany, France	L. Le Gall, J.M. Utge, F. Rousseau	CNEM001-10
Liagoraceae				
Cumagloia andersonii (Farlow) Setchell *et* Gardner	GWS001399	Bamfield, Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC5750-09
Cumagloia andersonii	GWS002881	Bamfield, Sparlingia Pt., Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC653-06
Cumagloia andersonii	GWS002882	Bamfield, Sparlingia Pt., Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC654-06
Cumagloia andersonii	GWS003158	Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, R. Withall	ABMMC835-06
Cumagloia andersonii	GWS003219	Bamfield, Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC1078-06
Cumagloia andersonii	GWS004303	Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1096-06
Cumagloia andersonii	GWS004304	Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1309-07
Cumagloia andersonii	GWS004305	Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1310-07
Cumagloia andersonii	GWS004393	Botanical Beach, Port Renfrew, Vancouver I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1312-07
Cumagloia andersonii	GWS004593	Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1104-06
Cumagloia andersonii	GWS009153	Waypoint #45 from Land's End to Pachena Beach, Bamfield, British Columbia, Canada	G. Saunders, B. Clarkston	ABMMC2198-08
Cumagloia andersonii	GWS021723	Soberanes Point, California, USA	B. Clarkston, K. Hind, S. Toews	ABMMC11691-10
Cumagloia andersonii	GWS021922	Santa Cruz (Four Mile), California, USA	B. Clarkston, K. Hind, S. Toews	ABMMC12110-10
Helminthocladia calvadosii (J.V. Lamouroux *ex* Duby) Setchell	LLG3069	Beniguet, Finistère, Brittany, France	Y. Gladu, A. Besnier, L. Le Gall	CNEM002-10
Helminthocladia hudsonii (C. Agardh) J. Agardh	LLG3000	Lampaul île Ségal, Finistère, Brittany, France	L. Le Gall, J.M. Utge, Y. Turpin	CNEM003-10
Helminthora divaricata (C. Agardh) J. Agardh	LLG3071	Beniguet, Finistère, Brittany, France	Y. Gladu, A. Besnier, L. Le Gall	CNEM004-10

Table 1. List of samples used in this study with collection details and BOLD accession numbers *(cont'd)*

Taxa	*Voucher*	*Collection details*	*Collectors*	*BOLD Accession numbers*
Liagora distenta (Mertens *ex* Roth) J.V. Lamouroux	LLG2498	Anse ouest de la pointe du Cognet, Port-Cros, French Riviera, France	L. Le Gall, J.M. Utge	REDEU048-09
Liagora distenta	LLG2618	Pointe de Gueretion, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU060-09
Liagora distenta	LLG2634	Pointe de Gueretion, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU062-09
Liagora distenta	LLG2848	La Gabinière, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU095-09
Liagora distenta	LLG2851	La Gabinière, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU096-09
Liagora pectinata F.S. Collins *et* Hervey	CWS06-14-5	Due south of Frick's Beach, Tucker's Town, Bermuda, USA	C.W. Schneider, C.E. Lane	ABMMC3693-09
Liagora viscida (Forsskål) C. Agardh	LLG2593	Montremian, Port-Cros, French Riviera, France	L. Le Gall, J.M. Utge	REDEU058-09
Liagora viscida	LLG2856	La Gabinière, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU097-09
Nemalion helminthoides (Velley) Batters	LBC0041	Lastres playa, Spain	L. Bittner, F. Mineur	ABMMC2624-08
Nemalion helminthoides	TJS0066	St-Michel-de-Plouguerneau, Brittany, France	T. Silberfeld	ABMMC2626-08
Nemalion multifidum (F. Weber *et* D. Mohr) Chauvin	CWS09-38-6	Quonochontaug Central Beach, Charleston, Rhode Island, United States	C.W. Schneider	ABMMC4417-09
Nemalion multifidum	GWS001816	Mullaghmore Head, Connacht, Ireland	G. Saunders	ABMMC5798-09
Nemalion multifidum	GWS001817	Mullaghmore Head, Connacht, Ireland	G. Saunders	ABMMC5810-09
Nemalion multifidum	GWS001823	Spiddal, Connacht, Ireland	G. Saunders	ABMMC1064-06
Nemalion multifidum	GWS007440	Alexander Bay Causeway near Terra Nova Park, Newfoundland and Labrador, Canada	L. Le Gall, H. Kucera, D. McDevit	ABMMC6431-10
Nemalion multifidum	GWS007769	Bras dOr Lake entrance (bridge highway 105 near exit 13), Nova Scotia, Canada	L. Le Gall, H. Kucera, D. McDevit	ABMMC2563-08
Nemalion multifidum	GWS007770	Bra dOr Lake entrance (bridge highway 105 near exit 13), Nova Scotia, Canada	L. Le Gall, H. Kucera, D. McDevit	ABMMC655-06
Nemalion multifidum	GWS007771	Bras dOr Lake entrance (bridge highway 105 near exit 13), Nova Scotia, Canada	L. Le Gall, H. Kucera, D. McDevit	ABMMC959-06

Table 1. List of samples used in this study with collection details and BOLD accession numbers *(cont'd)*

Taxa	*Voucher*	*Collection details*	*Collectors*	*BOLD Accession numbers*
Nemalion multifidum	GWS008007	Pointe Sapin, Northumberland Straight, New Brunswick, Canada	L. Le Gall, H. Kucera, J.M. Utge	ABMMC1348-07
Nemalion multifidum	GWS008020	Pointe Sapin, Northumberland Straight, New Brunswick, Canada	L. Le Gall, H. Kucera, J.M. Utge	ABMMC1349-07
Nemalion multifidum	TJS0043	Ile de Batz, Brittany, France	T. Silberfeld	ABMMC2625-08
Nemalion multifidum	LLG1503	Les haies de la conchée, Saint-Malo, Ille-et-Vilaine, Brittany, France	L. Le Gall, F. Rousseau	CNEM005-10
Nemalion multifidum	LLG1594	Les haies de la conchée, Saint-Malo, Ille-et-Vilaine, Brittany, France	L. Le Gall, J.M. Utge, F. Rousseau	CNEM006-10
Nemalion lubricum Duby	LLG0143	Platga els Castell, Palamos, Catalunya, Spain	L. Le Gall	ABMMC1351-07
Nemalion sp.	GWS002647	Bridport, Tasmania, Australia	G. Saunders	ABMMC2071-08
Nemalion vermiculare Suringar	GWS002254	Bamfield, Dixon I., British Columbia, Canada	C. Bates	ABMMC656-06
Nemalion vermiculare	GWS002255	Bamfield, Dixon I., British Columbia, Canada	C. Bates	ABMMC5754-09
Nemalion vermiculare	GWS002261	Bamfield, Seppings I., British Columbia, Canada	G. Saunders	ABMMC657-06
Nemalion vermiculare	GWS002267	Bamfield, Blowhole at Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC5766-09
Nemalion vermiculare	GWS002794	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, S. Clayden	ABMMC658-06
Nemalion vermiculare	GWS002805	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, S. Clayden	ABMMC659-06
Nemalion vermiculare	GWS003220	Bamfield, Bradys Beach, British Columbia, Canada	G. Saunders	ABMMC5795-09
Nemalion vermiculare	GWS003485	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC5749-09
Nemalion vermiculare	GWS003486	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC5761-09
Nemalion vermiculare	GWS004017	Bamfield, Blowhole at Bradys Beach, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC5740-09
Nemalion vermiculare	GWS004031	Bamfield, Blowhole at Bradys Beach, British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC857-06
Nemalion vermiculare	GWS004722	Raft Cove, Vancouver Island, British Columbia, Canada	B. Clarkston, D. McDevit	ABMMC6015-09

Table 1. List of samples used in this study with collection details and BOLD accession numbers *(cont'd)*

Taxa	*Voucher*	*Collection details*	*Collectors*	*BOLD Accession numbers*
Scinaiaceae				
Scinaia complanata (F.S. Collins) A.D. Cotton	LLG2746	La Gabinière, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	CNEM007-10
Scinaia complanata	LLG2750	La Gabinière, Port-Cros, French Riviera, France	F. Rousseau, J.M. Utge	REDEU077-09
Scinaia confusa (Setchell) Huisman	GWS000056	Monterey, California, United States	G. Saunders	ABMMC6025-10
Scinaia confusa	GWS001642	Bamfield, Dixon I., British Columbia, Canada	G. Saunders	ABMMC5762-09
Scinaia confusa	GWS001659	Bamfield, opening of Grappler Inlet, British Columbia, Canada	G. Saunders	ABMMC5786-09
Scinaia confusa	GWS002768	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, S. Clayden	ABMMC1072-06
Scinaia confusa	GWS002817	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, S. Clayden	ABMMC1073-06
Scinaia confusa	GWS003007	Bamfield, Black Fish I., near Helby I., British Columbia, Canada	G. Saunders, J. Mortimer	ABMMC1077-06
Scinaia confusa	GWS003905	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1089-06
Scinaia confusa	GWS003906	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit	ABMMC1293-07
Scinaia confusa	GWS006529	Tahsis Narrows, British Columbia, Canada	B. Clarkston, D. McDevit, S. Hamsher	ABMMC1989-07
Scinaia confusa	GWS006554	Tahsis Princesa Channel, British Columbia, Canada	B. Clarkston, D. McDevit, K. Roy, S. Hamsher	ABMMC1990-07
Scinaia confusa	GWS006591	Tahsis Nuchatliz Island, (#37 on Esperenza Inlet Chart), British Columbia, Canada	B. Clarkston, D. McDevit, K. Roy, S. Hamsher	ABMMC5965-09
Scinaia confusa	GWS008171	Bamfield, Scotts Bay, British Columbia, Canada	D. McDevit, B. Clarkston, K. Roy, S. Hamsher	ABMMC6565-10
Scinaia confusa	GWS008734	Bamfield, Wizard I., British Columbia, Canada	G. Saunders, B. Clarkston, D. McDevit, K. Roy	AB 6682-10
Scinaia confusa	GWS009096	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston	ABMMC2184-08
Scinaia confusa	GWS010312	Tahsis, Mozina Pt., British Columbia, Canada	G. Saunders, B. Clarkston	ABMMC6865-10

Table 1. List of samples used in this study with collection details and BOLD accession numbers *(cont'd)*

Taxa	*Voucher*	*Collection details*	*Collectors*	*BOLD Accession numbers*
Scinaia confusa	GWS010532	Bamfield, Dixon I. (up-channel bay), British Columbia, Canada	B. Clarkston	ABMMC6942-10
Scinaia confusa	GWS010590	Bamfield, Wizard I., British Columbia, Canada	K. Hind, D. McDevit	ABMMC6952-10
Scinaia confusa	GWS010619	Bamfield, Scotts Bay, British Columbia, Canada	B. Clarkston, S. Toews	ABMMC4440-09
Scinaia confusa	GWS010706	Bamfield, Dixon I., British Columbia, Canada	G. Saunders, B. Clarkston	ABMMC6971-10
Scinaia confusa	GWS012573	Chaatl Island across from Newton Point, Haida Gwaii, British Columbia, Canada	G. Saunders, D. McDevit	ABMMC3929-09
Scinaia confusa	GWS012574	Chaatl Island across from Newton Point, Haida Gwaii, British Columbia, Canada	G. Saunders, D. McDevit	ABMMC3930-09
Scinaia confusa	GWS019539	Bamfield, Wizard I., British Columbia, Canada	G. Saunders, K. Dixon	ABMMC12860-10
Scinaia confusa	GWS019667	Bamfield, Scotts Bay, British Columbia, Canada	G. Saunders, K. Dixon	ABMMC12977-10
Scinaia confusa	GWS020963	Between Wiah Point & Cape Edensaw (#2), NW of Masset, Haida Gwaii, British Columbia, Canada	G. Saunders K. Dixon	ABMMC14003-10
Scinaia confusa	GWS020982	Between Wiah Point & Cape Edensaw (#2), NW of Masset, Haida Gwaii, British Columbia, Canada	G. Saunders, K. Dixon	ABMMC14020-10
Scinaia confusa	GWS021011	Between Wiah Point & Cape Edensaw (#2), NW of Masset, Haida Gwaii, British Columbia, Canada	G. Saunders, K. Dixon	ABMMC14046-10
Scinaia furcellata (Turner) J. Agardh	LLG0400	Gijon, Spain	L. Le Gall	ABMMC2882-08
Scinaia interrupta (A.P. de Candolle) M.J. Wynne	TJS0019	Roscoff, France	T. Silberfeld	ABMMC2884-08
Scinaia interrupta	GWS019633	Bamfield, opening of Grappler Inlet, British Columbia, Canada	G. Saunders, K. Dixon	ABMMC12945-10
Trichogloea herveyi W.R. Taylor	CWS06-14-4	Due south of Frick's Beach, Tucker's Town, Bermuda, USA	C.W. Schneider & C.E. Lane	ABMMC3692-09
Whidbeyella cartilaginea Setchell et N.L. Gardner	GWS019662	Bamfield, Scotts Bay, British Columbia, Canada	G. Saunders, K. Dixon	ABMMC12972-10

transverse sections. Sections were stained in an acidified aniline blue solution (10 parts 1% aniline blue solution added to 90 parts 7% acetic acid solution (Kraft, 1988)), then rinsed and permanently mounted in a 60% aqueous karo solution (4% formaldehyde to prevent microbial growth). Photomicrographs were obtained using a Leica digital camera (DFC480) mounted to a Leica microscope (CTR5000). All images were imported into Adobe®PhotoShop®5.5 for plate assembly.

Molecular analyses

DNA was extracted with a protocol modified from Saunders (1993) (see Saunders, 2005 for the modifications). The 5' end of the COI gene (COI-5P barcode region) was PCR amplified following protocol of Saunders (2005). PCR products were cleaned with the ExoSAP-IT reagent (USB Corporation, Cleveland, Ohio). The BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems (ABI), Foster City, California) was used for the sequencing of PCR products and reactions were analyzed on an ABI Prism 3100 genetic analyzer. Complete sequences (excluding the 5' and 3' PCR regions) were determined in both directions for all taxa and assembled using Sequencher TM 4.2 (Gene Codes Corporation, Michigan).

Sequence alignments and phylogenetic analyses

The COI-5P sequences were uploaded to the Barcode of Life Data System (BOLD; Ratnasingham & Herbert, 2007). A multiple sequence alignment was constructed in MacClade version 4.06 (Maddison & Maddison, 2003) and the final alignment included 664 bp for 87 collections (86 ingroup and *Palmaria palmata* (Linnaeus) Weber *et* Mohr). All analyses were conducted in PAUP* version 4.0b10 (Swofford, 2003). Cluster analyses were performed using uncorrected distances and the UPGMA algorithm (Sokal & Michener, 1958). An LSU alignment was built to infer phylogenetic relationships among the Nemaliales using 42 previously published sequences (see legend to Figure 1 for sources), as well as newly acquired data for (*Helminthocladia calvadosii* (J.V. Lamouroux *ex* Duby) Setchell (LLG3069/ FR715031), *Helminthocladia hudsonii* (C. Agardh) J. Agardh (LLG3000/FR715030), *Helminthora divaricata* C.Agardh) J.Agardh (LLG3071/ FR715032), *Nemalion helminthoides* (TJS0066/ FR715029), *Nemalion lubricum* (LLG0143/ FR715028), *Nemalion multifidum* (GWS001817/ FR715027), *Scinaia furcellata* (LLG0400/ FR715026) and *Whidbeyella cartilaginea* (GWS019662/ XXXXX). Introns and highly variable region were excluded, generating an alignment of 2911 bp. MrBayes MPI version 3.1.2 for Unix / Linux clusters (Huelsenbeck *et al.*, 2001) was used to complete bayesian inference under a general-time-reversible model. Sampling was performed every 1000 generations, and each run was replicated twice. Analyses were run for two million generations and an appropriate burn-in for each run was determined by plotting the overall likelihood against generations prior to estimating the posterior probability distribution. Final results were based on the pooled samples from the stationary phase of the two independent runs.

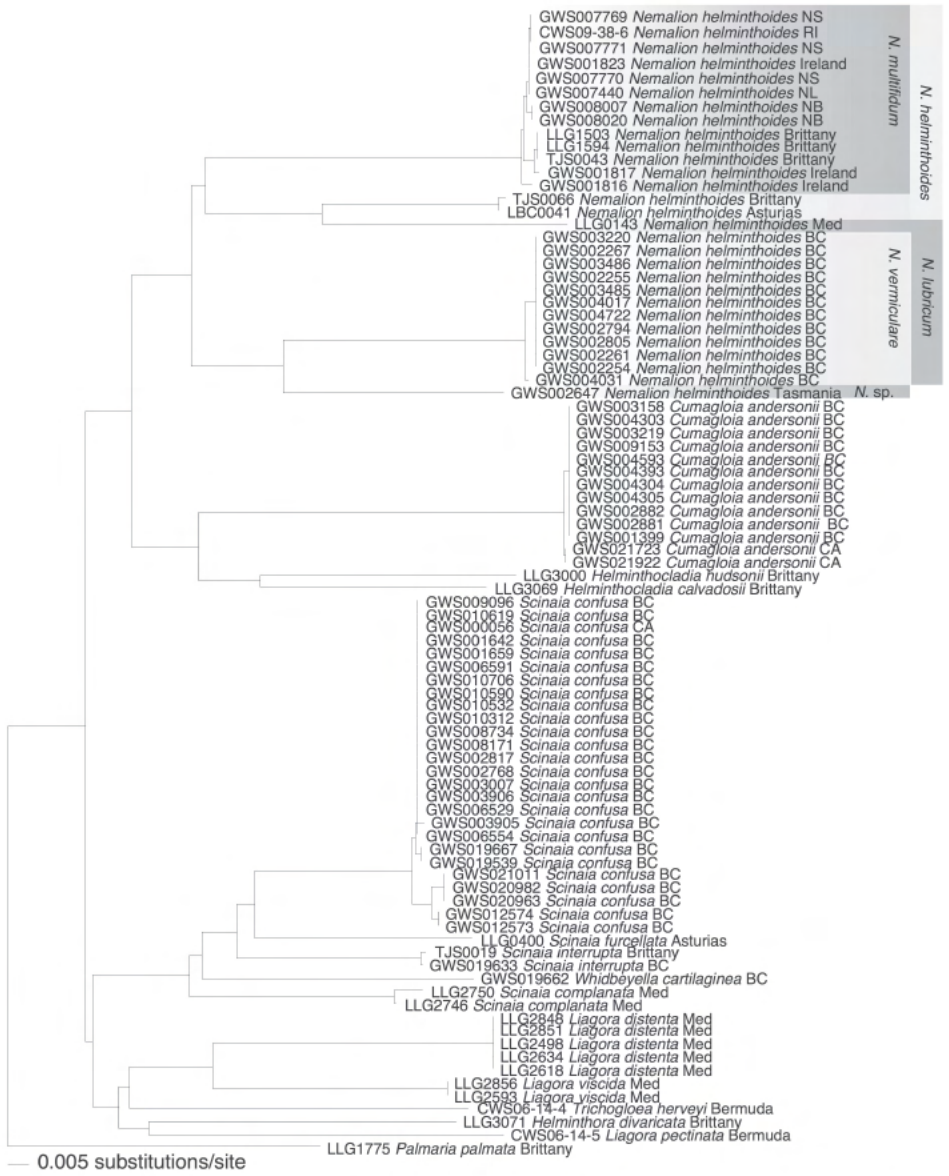


Fig. 1. Phylogram (neighbor joining) inferred from the DNA barcode sequences showing relationships among the 86 nemalialean specimens included in the present study. Voucher numbers and the geographical origin of each specimen are indicated. BC: British Columbia, CA: California, Med: Mediterranean, NB: New Brunswick, NL: Newfoundland, NS: Nova Scotia, RI: Rhode Island.

RESULTS AND DISCUSSION

Molecular results

In the present study, we generated COI-5P data for 86 nemalialean specimens and the tree resulting from cluster analyses is presented (Fig. 1). Considering that specimens with genetic distances less than or equal to 1% typically belong to the same species, our analyses resolved 18 nemalialean genetic groups that were divergent by 8% (*Scinaia confusa* and *S. turgida*) to 23% (*Liagora distenta* and *Nemalion lubricum*). Within a lineage, the highest variation (1%) was found among collections of *Scinaia confusa* from British Columbia. Intrageneric variation ranged from 8% within *Scinaia* to 18% in *Nemalion.* Interestingly, all specimens of the later taxon were field collected as *Nemalion helminthoides,* but resolved in our analysis as five distinct lineages (Fig. 1). Two lineages consisted of a single collection each from the Mediterranean Sea and Tasmania. A third lineage consisted of samples from the north Atlantic (Atlantic provinces of Canada, New England, Ireland and Brittany), while a fourth included a specimen from Brittany and a collection from Asturias. The fifth lineage included all samples from the Pacific coast of Canada.

The only other anomalous result was the identification of two species of *Scinaia* from the west coast of Canada (Fig. 1) where only one is reported (Gabrielson *et al.*, 2006). In addition to the widespread and expected *S. confusa*, we also uncovered a single crust (GWS019633; presumably the sporophytic stage) of the European *S. interrupta,* greatly extending the known range for this species. Whether this collection represents an introduction of this species to British Columbia or reflects its natural range will require substantially more sampling to resolve.

The phylogenetic relationships inferred from the large subunit of the ribosomal cistron (Fig. 2) are largely congruent with those published by Huisman *et al.* (2004a) in that the three families were resolved as monophyletic with full support. Interestingly, *Whidbeyella cartilaginea,* which is included here for the first time in molecular analyses, joins the genera *Scinaia* and *Nothogenia*, Scinaiaceae, as expected based on morpho-anatomical features (Huisman *et al.*, 2004a). All of the genera included, excepting *Liagora,* were resolved as monophyletic with the genus *Nemalion* sister to the remaining Liagoraceae with full support. *Liagora* has recently been split into several new or resurrected genera (Yoshizaki, 1987; Huisman & Schils, 2002; Huisman *et al.*, 2004b, Huisman *et al.*, 2006); however, our phylogeny reveals that substantial work is still needed to render this genus monophyletic.

Taxonomic results

The DNA-barcode approach has already proven a powerful ally in recognizing overlooked red-algal diversity (e.g. Saunders, 2008; Clarkston & Saunders, 2010; Le Gall & Saunders, 2010; Saunders & McDonald, 2010). The present study establishes that the supposedly cosmopolitan species *Nemalion helminthoides* actually encompasses multiple distinct genetic lineages, which are all characterized by relatively restricted biogeographical distribution patterns. Prior to our work, the genus *Nemalion* was considered to contain only seven species (Guiry & Guiry, 2010), but it has a convoluted history.

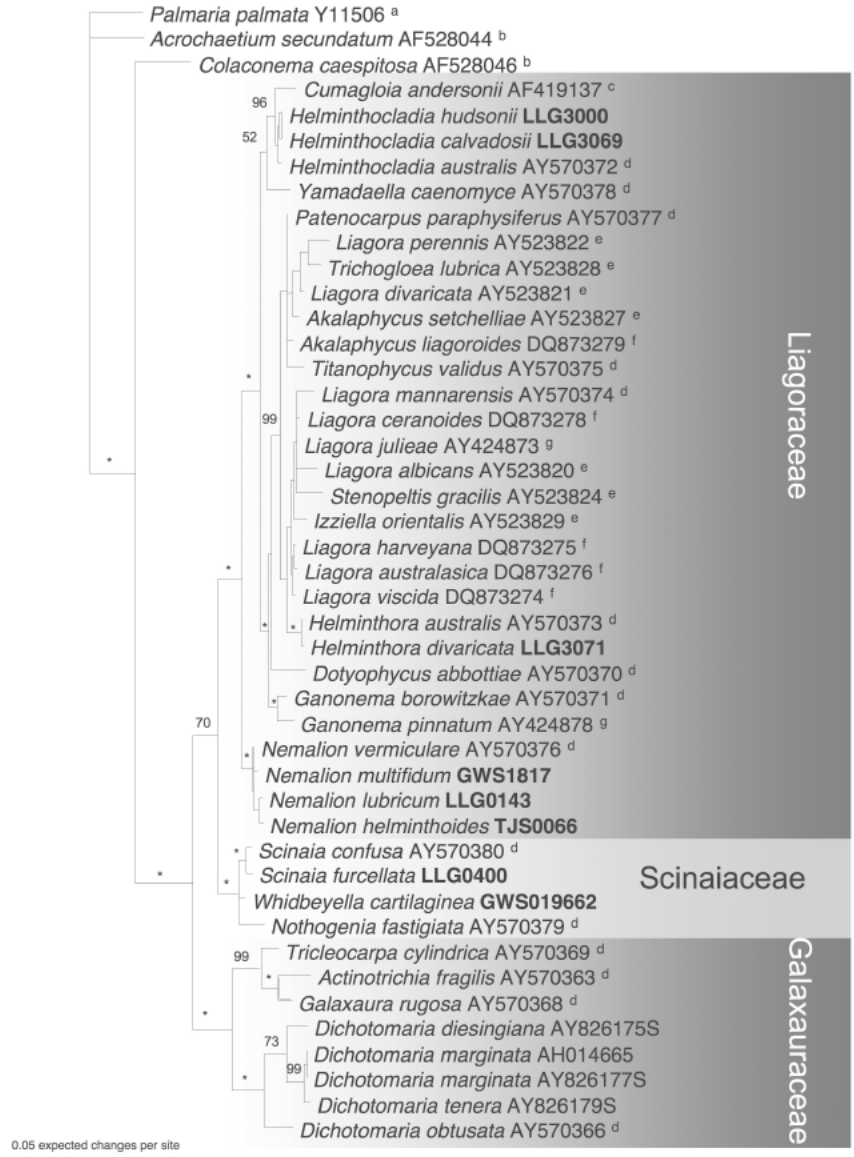
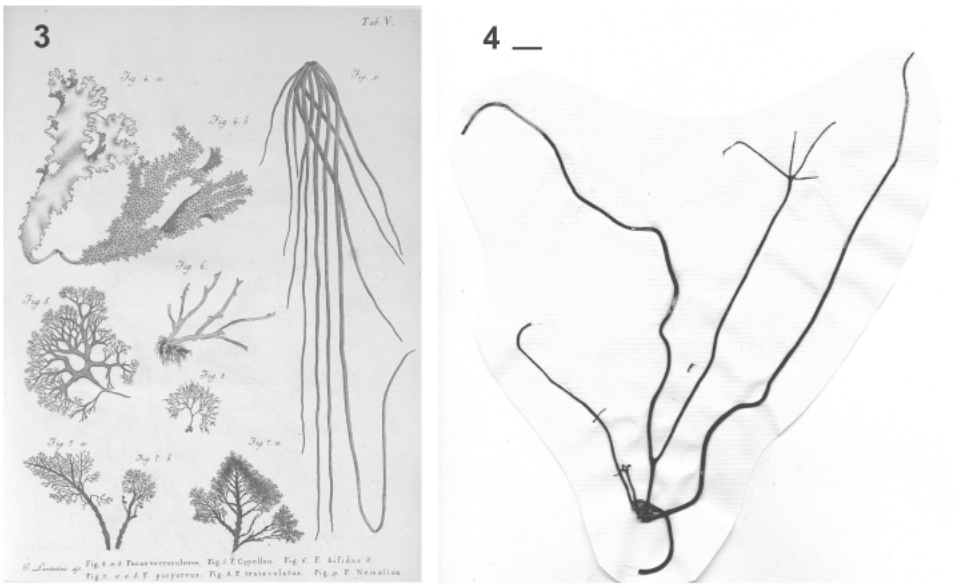


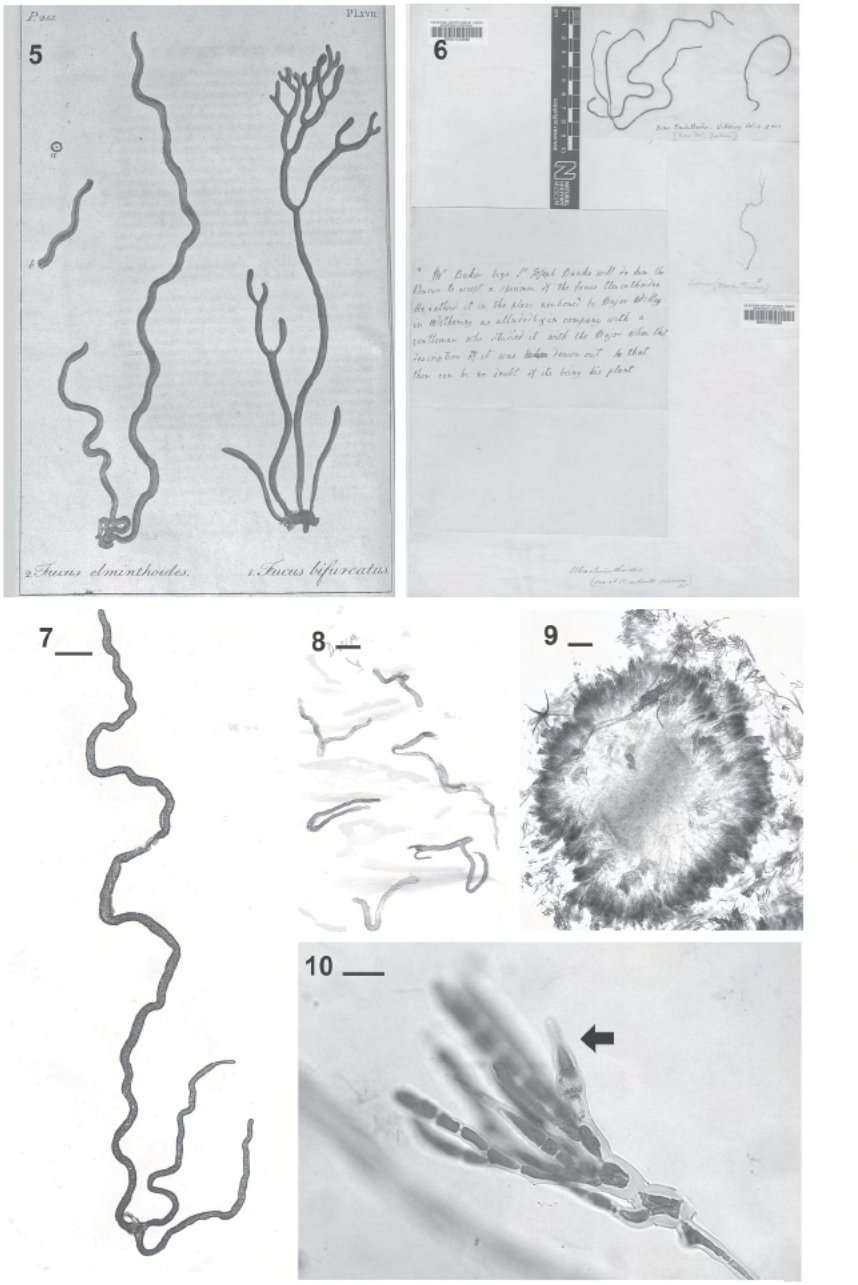
Fig. 2. Unrooted phylogram inferred from bayesian analyses of LSU data. Values above nodes indicate bayesian posterior probabilities. Voucher numbers in bold indicate sequences generated in the present study. Genbank accession numbers followed by a, b, c, d, e, f, g were generated by Van der Auwera *et al.* (1998), Harper & Saunders (2002), Harper & Saunders (2001), Huisman *et al.* (2004a), Huisman *et al.* (2004b), Huisman *et al.* (2006); Huisman *et al.* (2004c), respectively. * indicates 100% posterior probability.

Bertoloni (1818, 1819) described *Fucus nemalion* for specimens collected in San Terenzo (Gulf of Genoa) and provided an illustration (Fig. 3). Duby (1830) elevated the epithet '*nemalion*' to the generic level and proposed the nomen novum *Nemalion lubricum* considering the Mediterranean *Fucus nemalion* Bertoloni as the basionym. Despite the fact that the name *Nemalion lubricum* was widely used for both Atlantic and Mediterranean specimens of *Nemalion*, Batters (1902) corrected the epithet to *Nemalion elminthoides* (Velley) Batters granting authority to an older available name. The spelling of this specific epithet was changed to *helminthoides* in accordance with the ICBN recommendation (McNeill *et al.*, 2006, recommendation 60A2) that the spiritus asper (rough breathing) of Greek should be transcribed as the letter 'h'. Analysis of barcode data clearly indicates that our Mediterranean collection of *Nemalion* differs from Atlantic cogenors. The type of *Nemalion lubricum* Duby was depicted (Fig. 3) by Bertoloni (1818, 1819) and is morphologically similar to our Mediterranean specimen (Fig. 4). We therefore resurrect *Nemalion lubricum* Duby for the Mediterranean species of *Nemalion*.

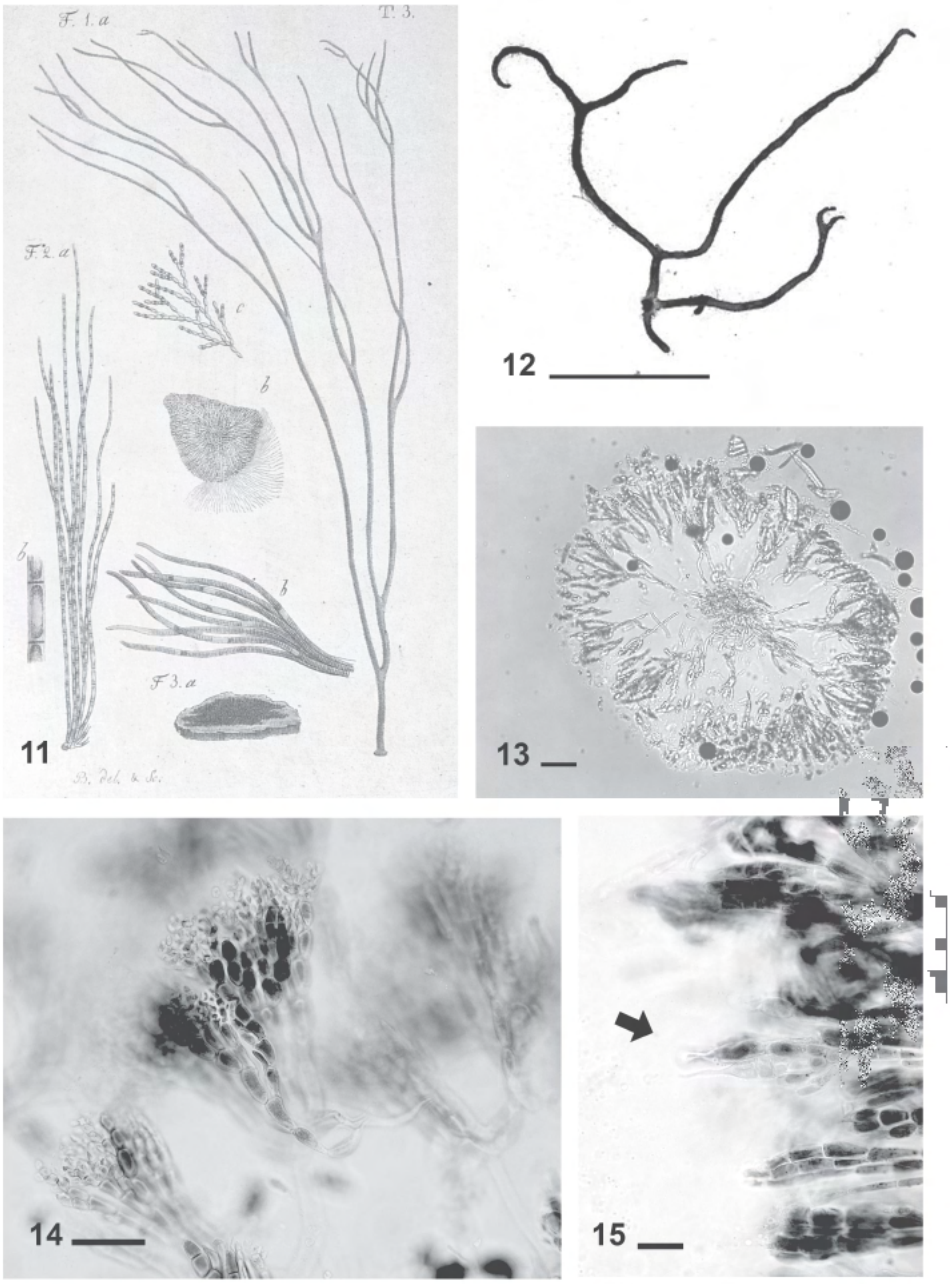
In 1792, Velley described and illustrated (Fig. 5) *Fucus elminthoides* in Withering's book entitled "A botanical arrangement of British plants" based on a specimen (BM001023865; Fig. 6) currently hosted in BM after its recent transfer from K (abbreviation in accordance with Holmgren *et al.* 1990). About a decade later Weber & Mohr (1804) described and illustrated (tabulae 3) (Fig. 11) specimens collected in Varberg along the Sweden coast of the Kattegat as "*Rivularia multifida*", a taxon that was later transferred to the genus *Nemalion* by J. Agardh (1841). Two specimens collected by Weber & Mohr in July 1803 and considered as isotypes (Botanical museum, Uppsala) were photographed by Söderström (1970). The latter author recognized both *Nemalion helminthoides* and *Nemalion multi-*



Figs 3-4. *Nemalion lubricum*. **3.** Illustration published by Bertoloni (1818) in Tab. V. *Nemalion lubricum* (as *Fucus nemalion*) collected in San Terenzo (Golf of Genoa) is illustrated on the right (Bertoloni's figure 9). **4.** Specimen (voucher LLG0143/PC0152544) collected in Catalunya, Spain. Scale bar 1 cm.



Figs 5-10. *Nemalion helminthoides*. **5.** Illustration published by Velley in Withering's book (1792) on PL. XVII p. 255. *Fucus helminthoides* (as *Fucus elminthoides*) collected in Portland, England is illustrated on the left (Velley's figure 2). **6.** Type specimen (BM001023865). **7.** Specimen (voucher TJS0066/PC0171239) collected in Brittany. Scale bar 1 cm. **8.** Specimen (voucher LBC0041/PC0152531) collected in Asturias. Scale bar 1 cm. **9.** Transverse section of LBC0041 showing the relatively thick medulla. Scale bar 100 µm. **10.** Squash of LBC0041 showing a carpogonium (arrow). Scale bar 30 µm.



Figs 11-15. *Nemalion multifidum*. **11.** Illustration published by Weber & Mohr (1804). *Nemalion multifidum* (as *Rivularia multifida*) collected in Varberg, Province of Halland, Sweden, is illustrated on the right (Weber & Mohr's figure 1a). **12.** Specimen (voucher LLG1594/PC0152528) collected in Brittany. **13.** Transverse section of LLG1594 showing the relatively small medulla. Scale bar 100 µm. **14.** Squash of GWS008020 showing spermatangia. Scale bar 30 µm. **15.** Squash of GWS008007 showing a carpogonium. Scale bar 30 µm.

fidum and argued that the two species can be distinguished by their branching pattern (*N. helminthoides* rarely branched above the base whereas *N. multifidum* is commonly branched), the shape of their apices (blunt in *N. helminthoides*, but tapering in *N. multifidum*), the thickness of the medulla relative to the cortex (*N. multifidum* having less central medulla than *N. helminthoides*), the color (dark brown to purple in *N. helminthoides*, red to blackish brown in *N. multifidum*) and their life cycle (*N. helminthoides* being dioecious whereas Söderström observed monoecious plants of *N. multifidum* in great Britain and France and dioecious ones in Sweden).

In light of our morphological observation of the type of *Nemalion helminthoides* (BM001023865, Fig. 6), characters that Söderström identified to distinguish the two species, and our molecular results, we assign our thicker and unbranched specimens collected in Brittany (Fig. 7) and Asturias (Figs 8-10) to *Nemalion helminthoides*. These specimens have a wide and dense medulla of axial filaments (Fig. 9) and the only fertile specimen (LBC0041) bears exclusively carpogonia (Fig. 10). In contrast, we assign to *N. multifidum* (Fig. 11) our collections of thinner and more branched specimens from Canada, Ireland and Brittany (Figs 12-15). These specimens have a thin medulla composed of loosely tangled axial filaments (Fig. 13). We observed specimens of *N. multifidum* being either exclusively male (Fig. 14) or female (Fig 15). Interestingly, our observation of a female for *Nemalion helminthoides* (LBC0041 Fig. 10) is congruent with Söderström's observation of dioecy for this species. However, our specimens of *N. multifidum* from Brittany are also dioecious whereas Söderström observed monoecious plants in this region. This difference can either be due to some variation in the life cycle of *Nemalion multifidum* or to the presence of another cryptic species that is monoecious. It is noteworthy than a fertile specimen of *N. lubricum* (LLG0143) bears only carpogonial branches suggesting that the later species is also dioecious.

In contrast, our Pacific collections of *Nemalion* were all monoecious when fertile. The only Pacific species of *Nemalion* described so far, *Nemalion vermiculare* Suringar, is also reported as being monoecious (Masuda, 1977). We therefore assign the Canadian Pacific species group to *N. vermiculare* pending further study of material from the type locality (Japan). The Australian specimen of *Nemalion* barcoded in the present study is likely to represent a new species; however, we refrain from describing it pending the study of additional collections from this region.

Acknowledgements. We gratefully acknowledge the collecting help of C. Bates, A. Besnier, L. Bittner, B. Clarkston, S. Clayden, K. Dixon, Y. Gladu, S. Hamsher, K. Hind, H. Kucera, C. Lane, D. McDevit, F. Mineur, J. Mortimer, F. Rousseau, K. Roy, C.W. Schneider, T. Silberfeld, S. Toews, Y. Turpin, J.M. Utge and R. Withall. Jo Wilbraham, curator of algal collections at the Natural History Museum is warmly thanked for providing the picture of *N. helminthoides*. We would like to thank Céline Bonillo, Dan McDevit, Tanya Moore and Jose Maria Utge for their technical support. Insights concerning the nomenclature of *Nemalion lubricum* from Bruno Dennetière were greatly appreciated. We are grateful to the Bamfield Marine Sciences Centre for hosting a large component of the fieldwork for this study. We thank Dr Norm Sloan for encouraging us to study the seaweeds of Haida Gwaii, as well as Parks Canada and the Haida Nation, especially staff of the Gwaii Haanas National Park Reserve and Haida Heritage Site, for extensive support of our field studies in this unique region. Particular appreciation is extended to Clint Johnson and Dan Bartol – our guides while in Gwaii Haanas. This study was supported by the "Service de systématique moléculaire", – UMS MNHN-CNRS 2700 of the Muséum national d'histoire naturelle. This research was supported through funding to the Canadian Barcode of Life Network from Genome Canada (through the Ontario Genomics Institute), the Natural Sciences and Engineering Research Council of Canada and other sponsors listed at www.bolnet.ca. The Canada Research Chair Program, the Canada Foundation for Innovation and the New Brunswick Innovation Foundation provided additional support.

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