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INTRODUCTION OF *GRACILARIA VERMICULOPHYLLA* (RHODOPHYTA, GRACILARIALES) TO NEW ENGLAND, USA: ESTIMATED ARRIVAL TIMES AND CURRENT DISTRIBUTION

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ABSTRACT. The invasive Asiatic red alga *Gracilaria vermiculophylla* has recently spread rapidly around the globe. In the Northwest Atlantic, it was first collected in Virginia during 1998; in New England, it was first recorded from Narragansett Bay, Rhode Island in 2007. Until now, the specific dates of its introduction and current distribution in New England have been poorly understood. We employed a combination of field collections, evaluations of historical herbarium specimens, and molecular investigations (including mt-CO1 gene sequencing) to document its present distribution and approximate dates of introduction within New England. We found *G. vermiculophylla* at 18 of 24 Northwest Atlantic sites growing with native *Gracilaria* populations. Presently, it is recorded from Stamford, CT to Greenland, NH, with no populations known from five Maine sites where the native *G. tikvahiae* grows. Molecular screening of historical specimens revealed that *G. vermiculophylla* was collected from five sites in Massachusetts during 2000, whereas it was first documented in New Hampshire from the middle of the Great Bay Estuarine System (i.e., Dover Point) during 2003. In Rhode Island, initial specimens were documented during 2007, and those in Connecticut were first confirmed during

2010. As *G. vermiculophylla* has gone primarily undetected in New England since at least 2000, this highlights the difficulty of documenting the arrival and spread of an invasive species that closely resembles a native congener. Hence, DNA sequencing is critical in clarifying the introduction and expansion of such non-native seaweeds.

Key Words: Asian, *Gracilaria vermiculophylla*, *Gracilaria tikvahiae*, introduction, invasive, expansion, New England, molecular confirmations

There are more than 120 known introduced seaweeds worldwide, with many of these causing environmental and economic harm (Mathieson et al. 2008; Nyberg and Wallentinus 2005). Twenty-five introduced seaweeds are known from the Northwest Atlantic, including three green, five brown, and 17 red algae (Hofmann et al. 2010; Mathieson et al. 2008; Schneider 2010; Thornber et al. 2009); many of these species were previously overlooked due to morphological similarities to native species. One such example is the native *Gracilaria tikvahiae* McLachlan and the morphologically similar congener *G. vermiculophylla* (Ohmi) Papenfuss. *Gracilaria vermiculophylla* has recently and rapidly spread around the globe (Rueness 2005), with introductions in the eastern Pacific (Bellorin et al. 2004; Saunders 2009), northeastern Atlantic including Denmark, Sweden (Nyberg 2007), Germany (Thomsen et al. 2007; Weinberger et al. 2008), and France (Rueness 2005), plus the Mid-Atlantic coast of North America (Freshwater et al. 2006; Mathieson et al. 2008; Thomsen et al. 2006) and New England (Saunders 2009; Schneider 2010; Thornber et al. 2009).

The speed of dispersal and subsequent success of *Gracilaria vermiculophylla* in these regions is notable. In the northeastern Atlantic, it was first detected in the Göteborg archipelago, Sweden during 2003, and in two years it spread to over 30 sites with a distributional range of 150 km (Nyberg 2007). A few years after its introduction to Germany and Denmark, it became the most abundant seaweed in many soft-bottom estuarine sites, particularly those with low salinities (Thomsen et al. 2007). A half decade after the French introduction, *G. vermiculophylla* was present in most estuaries of the Brittany and Iberian coasts, often forming extensive unialgal entangled mats (Abreu 2011; Rueness 2005; Saunders 2009). In the southeastern Gulf of California, it was frequently observed forming expansive blooms in coastal lagoons (Pinion-Gimate et al. 2009). In the Mid-Atlantic coast of North America, it

was first discovered in North Carolina in 2000, and by 2002 its extensive growth on gill nets and trawls hindered commercial fishing and fouled intake screens at the Brunswick Nuclear Plant (Freshwater et al. 2006). In Hog Island Bay, Virginia, *G. vermiculophylla* was the dominant seaweed from 1998 through 2002, making up 74% of the total biomass across all study sites and seasons (Thomsen et al. 2006). It has continued to have significant impacts on saltmarsh habitat complexity, species richness and abundance, nutrient availability, productivity, and trophic interactions at several Virginia sites (Thomsen et al. 2009). The first published records of *G. vermiculophylla* from New England were from Narragansett Bay, Rhode Island (Saunders 2009; Schneider 2010; Thornber et al. 2009), but these accounts gave no details regarding its regional distribution or initial dates of introduction. Hence, our present study has attempted to clarify such information for this invasion.

As with many successful invaders, *Gracilaria vermiculophylla* has broad tolerances to temperatures, salinities (Abreu 2011; Nyberg 2007), nutrients, sediment burial, and grazing (Abreu et al. 2011a, b; Thomsen and McGlathery 2007). It also grows extensively from fragments (Abreu et al. 2011a, b; Nyberg and Wallentinus 2009), and is capable of successfully colonizing and expanding in regions where its entire life history may not be expressed. Nyberg and Wallentinus (2009) also found that it could survive 175 days in moist conditions under total darkness, resuming exponential growth following a return to normal light, salinity, and immersion conditions. Hence, the species is well suited for long-distance transport in ballast water, on ship hulls, or ship decks and can survive long term burial in estuarine environments (Nyberg and Wallentinus 2009).

Detection of *Gracilaria vermiculophylla* may be difficult because it is morphologically very similar to other species, such as *G. tikvahiae*, a native species with which it often shares the same habitats. *Gracilaria* species have highly plastic morphologies and exhibit subtly distinguishing vegetative features when growing under ideal conditions (Gurgel et al. 2004; Rueness 2005; Saunders 2009). In the absence of sexual characteristics, a common state in *Gracilaria* (Rueness 2005), molecular analysis is the most useful method of species identification (Saunders 2009; Thomsen et al. 2006). In order to determine the current distribution and approximate introduction times of *G. vermiculophylla* in New

England, we used a three-pronged approach, involving field sampling, investigations of historical collections, and molecular identifications of specimens by comparing sequences of the mt-CO1 gene. This region was selected due to its demonstrated usefulness as a barcode for certain red algal groups (Saunders 2009).

MATERIALS AND METHODS

Between 2007 and 2011, we surveyed 24 New England estuarine sites having known *Gracilaria* spp. populations, ranging from western Connecticut to mid-coastal Maine (Table 1; Figure 1). In addition, we surveyed eight other comparable New England sites with no previous *Gracilaria* records (Wilbur Neck, Pembroke, ME; Little Augusta River, Whiting, ME; Winslow Park, South Freeport, ME; Nubble Lighthouse, York, ME; Damons Point, Marshfield, MA; Niantic, CT; Guilford Marina, Guilford, CT; and New Haven Lighthouse, New Haven, CT). Collections were made either on foot at low tide or by snorkeling at mid-tide. Depending on local abundance, between five and 15 *Gracilaria* thalli per site were collected for morphological and/or molecular analysis.

In the field, all collected specimens were initially rinsed *in situ* to remove sediments and then placed in labeled zip-lock bags for transfer to the lab where they were floated in seawater, pressed as voucher specimens, and deposited in the Hodgdon Herbarium (NHA) at the University of New Hampshire (UNH), or in the herbaria at the Universities of New Brunswick (UNB) or Rhode Island (KIRI). In addition to the fresh field collections, 48 voucher *Gracilaria* "*tikvahiae*" specimens (Appendix), collected by various researchers between 1966 and 2007 from sites in New England, were molecularly screened to determine species identifications and to approximate dates of *G. vermiculophylla* introduction using the following methods for DNA extraction, amplification, purification, sequencing, alignment, and GenBank comparison.

Gracilaria samples were ground in labeled 1.7 ml microcentifuge tubes using disposable plastic pestles, 10 mg of molecular grade sand, and 300 ml of Gentra Puregene® Cell Lysis Solution (D-5002). The DNA was extracted with a Gentra Puregene® Isolation Kit following manufacturer's instructions (Qiagen, Valencia, CA). Samples were incubated in a 65°C heatblock for one hour inverting 10 times at 30 min and cooled to room temperature before 100 µl of Protein Precipitation Solution (Gentra D-5003) were added.

Table 1. *Gracilaria* collections from 24 sites ranging from mid-coastal Maine to Stamford, Connecticut. GT and GV represent *G. tikvahiae* (native) and *G. vermiculophylla* (non-native), respectively. Dates given for *G. vermiculophylla* specimens indicate the date of the earliest molecularly verified collection. Dates given for non-invaded sites indicate the year of the most recent molecularly verified *G. tikvahiae* collection. [1]Dashes indicate range of accession numbers; the GWS samples are held at the University of New Brunswick herbarium (UNB).

Site	Location	Latitude °N	Longitude °W	Year Collected	Species	[1]Herbarium Accession #
1	Oyster Creek, Salt Bay, Damariscotta, ME	44°03′28″	69°30′34″	2010	GT	NHA554802
2	Salt Bay, Damariscotta, ME	44°03′00″	69°51′40″	2010	GT	NHA554806–7
3	Upper New Meadows River, Bath, ME	43°55′50″	69°31′41″	2010	GT	NHA554805
4	Pennellville Landing, Brunswick, ME	43°51′18″	69°57′39″	2010	GT	NHA554803–4
5	Merepoint Boat Launch, Brunswick, ME	43°49′42″	70°00′59″	2010	GT	NHA554809
6	Wharton Point, Maquoit Bay, Brunswick, ME	43°52′01″	69°59′33″	2010	GT	NHA554801
7	Dover Point, Little Bay, Durham, NH	43°07′15″	70°49′35″	2003	GV, GT	NHA554808
8	Sunset Farm, Great Bay, Greenland, NH	43°03′24″	70°50′03″	2008	GV, GT	NHA524468–9
9	Depot Road, Great Bay, Greenland, NH	43°03′22″	70°53′50″	2008	GV, GT	NHA554795
10	Lubberland Creek, Great Bay, Newmarket, NH	43°04′30″	70°54′12″	2008	GV, GT	NHA524474
11	Mattakeeset Town Landing, Duxbury, MA	42°02′22″	70°40′11″	2000	GV	NHA554785, NHA554798
12	Bluefish River, Shipyard Center, Duxbury, MA	42°02′47″	70°40′18″	2000	GV	NHA554787–8, NHA554799
13	Ellisville Harbor State Park, Plymouth, MA	41°50′28″	70°32′07″	2000	GV	NHA554796
14	Indian Trail Rd., Barnstable, MA	41°42′35″	70°17′02″	2011	GV	NHA554789
15	Millway Beach, Barnstable, MA	41°42′34″	70°17′59″	2011	GV	NHA554793–4
16	Provincetown Harbor, MA	42°02′58″	70°11′09″	2000	GV	NHA554786
17	Capt. Nathaniel Wixon Dock, W. Harwich, MA	41°39′29″	70°06′55″	2011	GV	NHA554792

Table 1. Continued.

Site	Location	Latitude °N	Longitude °W	Year Collected	Species	[1]Herbarium Accession #
18	Lewis Pond, Sea Gull Beach, W. Yarmouth, MA	41°38′12″	70°13′40″	2000	GV	NHA554790, NHA554797
19	Goddard State Park, Warwick, RI	41°40′03″	71°25′52″	2007	GV	NHA556929–30
20	Budlong Farm, Warwick, RI	41°41′10″	71°25′24″	2007	GV	NHA556931–3
21	Bass Rock, Narragansett, RI	41°24′18″	71°27′27″	2007	GV	NHA556985
22	Potter Pond, South Kingston, RI	41°22′56″	71°32′04″	2009	GV, GT	NHA556934
23	Seaside Beach, Bridgeport, CT	41°09′10″	73°12′39″	2010	GV	GWS022482–3
24	Holly Pond, Cove Island State Park, Stamford, CT	41°02′57″	73°30′08″	2010	GV, GT	NHA524712

Samples were inverted 150 times and chilled at −20°C for 45 min before they were centrifuged for 15 min at 13,000 rpm. For each sample, the supernatant was then poured into a new 1.7 ml microcentifuge tube containing 300 µl of 100% isopropanol and inverted 50 times before centrifugation for 10 min at 13,000 rpm. The alcohol was decanted and replaced with 300 µl of 70% ethanol before inversion and 5 min of centrifugation at 13,000 rpm. The alcohol was decanted, and the sample was air dried for 60 min before 50 µl of DNA Hydration Solution (Gentra D-5004) were added. After briefly mixing, the samples were incubated in a 65°C heatblock for one hour and centrifuged for 5 min.

Polymerase chain reactions were carried out in 50 µl volumes containing 4 µl extracted DNA, 10 µl Taq buffer (GoTaq® Flexi Green; Promega, Madison, WI), 0.2 mM Mg^{2+}, 1 µl dNTPs, 1 µl each (20 mM) primer, and 0.25 µl Taq polymerase (GoTaq® Flexi). The primers used for amplification and sequencing were CO1F328 (5′ ACA GGA TGA ACA GTK TAT CCY C 3′) and CO1R634 (5′ CCA CCT GCW GGA TCA AAG A 3′), with these being selected due to their compatibility with one another in terms of CG content, melting temperature, and primer dimer avoidance. Additionally, the resulting fragment length created by these primers led to a high success rate for the amplification of DNA from older specimens.

The PCR products were separated by electrophoresis on a SYBR®Safe (available from Life Technologies, Grand Island, NY) treated low-melt agarose gel (0.8%) in nTBE Buffer (0.5×). On a UV lightbox, the desired DNA bands were excised using microscope slide cover slips, and transferred to 1.7 ml tubes, incubated in a 65°C heatblock for 5 min, and transferred to a 37°C heatblock. To each tube, 1.5 µl of agarase (Sigma A6303, 50 units/ml) were added, and the mixture was incubated overnight.

Concentrations of DNA were quantified using an Invitrogen™ Quant-iT™ dsDNA BR Assay Kit (Q32851) and an Invitrogen™ Qubit™ fluorometer (Q32857), following manufacturer's instructions (Life Technologies). Appropriate volumes of DNA and primers were then sent to the Hubbard Genomic Center (UNH) for clean-up and sequencing reactions using Applied Biosystems BigDye Terminator Cycle Sequencing Kits (v1.1 and v3.1; available from Life Technologies). The DNA samples were resolved by capillary electrophoresis on an ABI3130 DNA Analyzer.

Resulting sequences were trimmed in Chromas (version 2.2; Technelysium, Pty. Ltd., Tewantin, Queensland, Australia). Sequence

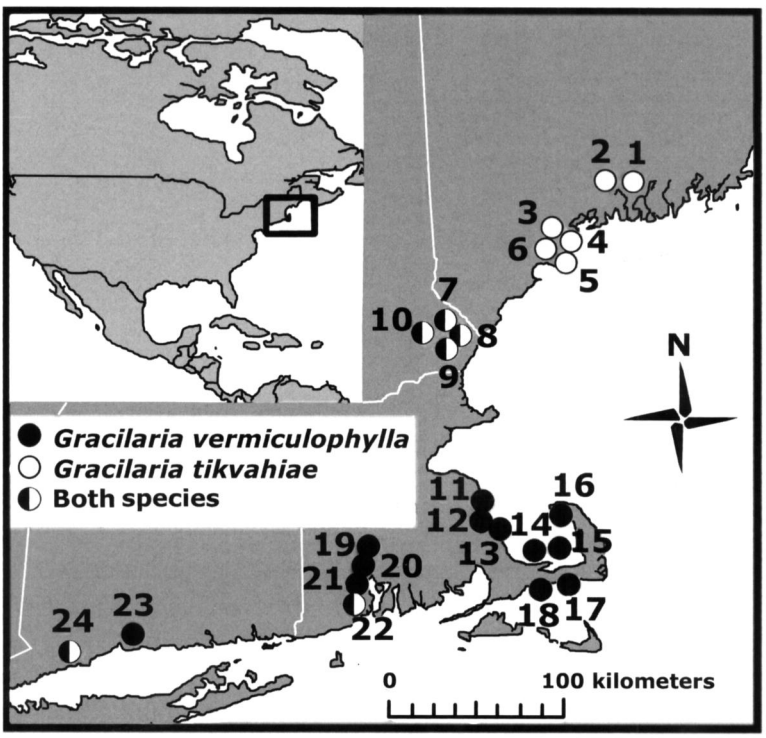

Figure 1. Distributions of *Gracilaria vermiculophylla* (non-native) and *G. tikvahiae* (native) in New England, based on samples collected from 2000 to 2011. The six Maine samples were collected in 2010. Site numbers correspond to those given in Table 1.

assembly and alignments were made and proofed using Seq Man II (version 7.1 for Windows; DNAStar, Inc., Madison, WI). Comparative alignments and GenBank searches were performed using MegAlign (version 7.1 for Windows; DNAStar, Inc.). Representative voucher specimen sequences were deposited in GenBank (accession numbers JQ675682–JQ675712, JQ699274–699286, and JQ716364–JQ716366).

The segment of DNA amplified was 307 bp in length, extending from position 328 of mt-CO1, a region of the mitochondrial genome commonly used as a barcode for distinguishing closely related species within certain groups of red algae (Saunders 2005). The 3′ end of the mt-CO1 DNA segment was used for our identifications because it amplified more readily in historical

collections than the 5′ end, and it consistently revealed differences between *Gracilaria* species with reference GenBank sequences. A divergence of 13% (41 bp) was evident between *G. tikvahiae* and *G. vermiculophylla* (J.C.N., pers. obs.).

RESULTS

Based upon DNA identifications of the mt-CO1 gene (matched to GenBank accessions FJ499599–FJ499628), we confirmed the presence of *Gracilaria vermiculophylla* at 18 of 24 New England sites (75%) known to have *Gracilaria* populations, with these ranging from Stamford, CT to Greenland, NH (Table 1, Figure 1, Appendix). This expands the documented range of this introduction by over 200 km north, 100 km east, and 150 km west. No *G. vermiculophylla* populations were found at any of the six Maine sites where *G. tikvahiae* was previously recorded, and no specimens of either *Gracilaria* species were found at any of the eight New England sites where *Gracilaria* populations were previously unknown. Mixed populations of *G. tikvahiae* and *G. vermiculophylla* were found at four of the New Hampshire sites, as well as in Potter Pond, South Kingston, RI, and Holly Pond, Stamford, CT. In the eight Massachusetts sites surveyed during March 2011, only *G. vermiculophylla* populations were found.

Gracilaria vermiculophylla was primarily found in estuarine sites having muddy or fine sandy bottoms. New Hampshire specimens were almost exclusively loose-lying or partially buried in sediment, whereas those from Massachusetts, Rhode Island, and Connecticut were typically attached to shells, small rocks, and other hard surfaces, with only occasional drifting specimens.

Triphasic life history patterns (i.e., male, female gametophytes, carposporophyte, and tetrasporophyte) of Rhode Island *Gracilaria vermiculophylla* populations have been confirmed (Thornber, pers. obs.). Such patterns suggest that the plant exhibits both sporic and vegetative fragmentation as means of reproduction.

Molecular screening of 48 historical specimens showed that the first New England *Gracilaria vermiculophylla* vouchers were collected at five Massachusetts sites in 2000 (Appendix). In New Hampshire, the first *G. vermiculophylla* specimen was collected in 2003 from Dover Point, within the middle of the Great Bay Estuarine System. An initial genetic confirmation of *G. vermiculophylla* from Rhode Island was made during 2007 (cf. Saunders 2009; Thornber et al. 2009). It was not found in Connecticut prior to 2010.

DISCUSSION

Based upon molecular analyses of historical collections, *Gracilaria vermiculophylla* has existed primarily undetected in New England since at least 2000, several years prior to the first published records (Saunders 2009; Schneider 2010; Thornber et al. 2009). Such findings confirm the difficulty of documenting the arrival and spread of an invasive species that closely resembles a native congener (e.g., *G. tikvahiae*). To complicate matters, both species can survive year-round within estuarine low intertidal/subtidal habitats, and they often grow together. For example, in Great Bay, New Hampshire, we frequently found vegetative specimens of both species within a single 0.25 m^2 quadrat frame. Although thallus length, cortical cell diameter, and cystocarp shape might be used to distinguish *G. vermiculophylla* from *G. tikvahiae* under ideal conditions, these characteristics are highly plastic and unreliable. As such, species determinations in the field are impossible, and DNA sequencing is essential for their identification, as with other cryptic introduced species in New England (Hofmann et al. 2010).

Since its initial collection in Virginia during 1998 (Thomsen et al. 2006), *Gracilaria vermiculophylla* has invaded and become established in disconnected estuaries across thousands of kilometers along the US Atlantic coastline. In the western Atlantic, its distribution now rivals that of *G. tikvahiae*, which occurs in estuarine environments from southern Mexico to the Canadian Maritime Provinces (Gurgel et al. 2004).

Although *Gracilaria vermiculophylla* has not been reported as a nuisance to any maritime industries in New England, its wide distribution (over 500 km) and abundance in some locations could cause ecological problems. Even at low density, its competitive nutrient uptake ability and large biomass can have a negative impact on the eelgrass, *Zostera marina* L. (Nyberg 2007; Wallentinus and Nyberg 2007), a species that is an important food source, nutrient cycler, and habitat for various invertebrates and juvenile fish. *Zostera* populations in the Great Bay Estuarine System, NH/ME, Narragansett Bay, RI, and Long Island Sound, CT have been threatened for several decades (Oviatt 2004; Short 1992; Yarish 2006), and the inevitable spread of *G. vermiculophylla* within these systems could hinder their recovery.

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APPENDIX

Vouchers of *Gracilaria* "*tikvahiae*" specimens, collected by various researchers between 1966 and 2007 from sites in New England, that were molecularly screened to determine species identifications. GT and GV represent *G. tikvahiae* and *G. vermiculophylla*, respectively. Great Bay Estuarine System is abbreviated as GBES. The GWS samples are held at the University of New Brunswick herbarium (UNB).

Location	Collector	Date	Species	Herbarium Accession #
Weeks Point, GBES, NH	*Searles, M.P.*	1966	GT	NHA4535
Sandy Point, GBES, NH	*Mathieson, A.C.*	1966	GT	NHA748
Weeks Point, GBES, NH	*Mathieson, A.C.*	1967	GT	NHA10183A
Welsh Cove, GBES, NH	*Hutchinson, B.*	1972	GT	NHA18191
Sullivan Bridge, GBES, NH	*Mathieson, A.C.*	1975	GT	NHA23944
Dover Point, GBES, NH	*Norall, T., M. Josselyn*	1975	GT	NHA24068
Goat Island, GBES, NH	*Mathieson, A.C.*	1976	GT	NHA27963
Moody Point, GBES, NH	*Costa, M., P. Fullerton*	1977	GT	NHA35822
Oyster River, GBES, NH	*Fullerton, P.*	1977	GT	NHA35218
Holly Pond, Stamford, CT	*Yarish, C.*	1979	GT	NHA556940
Dover Point, GBES, NH	*Turgeon, L.*	1983	GT	NHA48284
Damariscotta River, ME	*Penniman, C.*	1985	GT	NHA48948
Holly Pond, Stamford, CT	*Yarish, C.*	1986	GT	NHA556941
Welsh Cove, GBES, NH	*Mathieson, A.C.*	1993	GT	NHA50299
Dover Point, GBES, NH	*Gerwick, J.*	1993	GT	NHA63876
Salt Bay, Nobleboro, ME	*Mathieson, A.C., E. Hehre*	1994	GT	NHA53058
Oyster River, GBES, NH	*Mathieson, A.C.*	1994	GT	NHA54075
Glidden Point, Damariscotta, ME	*Mathieson, A.C., E. Hehre*	1995	GT	NHA58130
Meduncook River, ME	*Mathieson, A.C.*	1995	GT	NHA57001
Dover Point, GBES, NH	*Reynolds, N.B.*	1996	GT	NHA15551
Mere Point, Brunswick, ME	*Mathieson, A.C.*	1999	GT	NHA70056
Duxbury Public Pier, Duxbury, MA	*Mathieson, A.C.*	2000	GV	NHA72561
MA Maritime Academy, Bourne, MA	*Mathieson, A.C.*	2000	GT	NHA72355
Seagull Beach, West Yarmouth, MA	*Mathieson, A.C.*	2000	GV	NHA73768
Provincetown Harbor, MA	*Mathieson, A.C.*	2000	GV	NHA73062
Ellisville Harbor State Park, MA	*Mathieson, A.C.*	2000	GV	NHA75757
Duxbury Marsh, Duxbury, MA	*Mathieson, A.C.*	2000	GV	NHA72810
Holly Cove, Stamford, CT	*Yarish, C.*	2002	GT	NHA556942
Dover Point, GBES, NH	*Johnson, K.*	2003	GV	NHA78247
Dover Point, GBES, NH	*Mathieson, A.C.*	2005	GT	NHA78278

Appendix. Continued.

Location	Collector	Date	Species	Herbarium Accession #
Potter Pond, South Kingston, RI	*Thornber, C.*	2007	GT	NHA556936
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	NHA556931
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	NHA556932
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	NHA556933
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009254
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009255
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009256
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009257
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009258
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009259
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009260
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009261
Budlong Farm, Warwick, RI	*Thornber, C.*	2007	GV	GWS009262
Goddard State Park, Warwick, RI	*Thornber, C.*	2007	GV	GWS009263
Goddard State Park, Warwick, RI	*Thornber, C.*	2007	GV	NHA556929
Goddard State Park, Warwick, RI	*Thornber, C.*	2007	GV	NHA556930
Bass Rock, Narragansett, RI	*Thornber, C.*	2007	GV	NHA556935
Potter Pond, South Kingston, RI	*Thornber, C.*	2007	GV	NHA556934