THE EVOLUTION OF THYASIRID CLAMS (BIVALVIA: THYASIRIDAE) FROM A SUBARCTIC FJORD IN BONNE BAY, NEWFOUNDLAND

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THE EVOLUTION OF THYASIRID CLAMS (BIVALVIA: THYASIRIDAE) FROM A SUBARCTIC FJORD IN BONNE BAY, NEWFOUNDLAND

by

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Abstract

Thyasirids (Bivalvia: Thyasiridae) are among the few bivalve families containing both symbiotic and asymbiotic species, making them an interesting study group from an evolutionary perspective. This work characterizes thyasirids from a subarctic fjord in the Northwest Atlantic (Bonne Bay, Newfoundland) using both morphological and molecular analyses, and assesses the host-specificity and co-speciation between thyasirids and symbionts. Specifically, we describe a species (*Thyasira Parathyasira* sp.) appearing to be distinct from previously described thyasirids, *T. P. equalis* and *T. P. dunbari*, suggesting it may be a new species. Further, *T. Parathyasira* sp. was not found to possess symbionts. We also are the first to describe a species complex in the Thyasiridae family, composed of at least two closely related, sympatric species (*Thyasira* aff. *gouldi*). We demonstrate that closely related species can possess wide morphological and symbiosis-related disparity, suggesting that chemosymbiosis may be important for setting up barriers of gene flow. Finally, we demonstrate low host-specificity and lack of co-speciation between thyasirids and their extracellular bacterial endosymbionts, suggesting that symbiosis is most likely opportunistic in the family.

Acknowledgements

Most importantly, I would like to thank my supervisor, Dr. Suzanne Dufour, who gave me the opportunity to conduct the following Master's project, and guided me through the entire process. I am thankful for the countless hours she spent not only editing the thesis, but also listening to my ideas and giving me valuable advice that has been incorporated into this research. She was always supportive and encouraging, making this project enjoyable overall. She also supported me financially through her Natural Sciences and Engineering Research Council of Canada Discovery Grant, in addition to the Ocean Industries Student Research Award I received from the Research and Development Corporation. I would also like to thank my thesis committee, Dr. Paul Snelgrove and Dr. Duncan McIlroy, who provided insight throughout the two-year project, and were extremely helpful with revising the thesis.

Other integral people to this project would include J. Laurich, who provided all of the images of gill sections (esp. in Chapter 2). R. Hooper assisted with thyasirid collection at the Bonne Bay Marine Station; E. Perry and E. Yaskowiak assisted with work performed at the CREAIT Genomics and Proteomics Facility (MUN). H.D. Marshall and D. Innes provided advice on the phylogenetic analysis, and S. Tucker and K. Williams (EM Unit, Faculty of Medicine, MUN) assisted with Transmission Electron Microscopy conducted by J. Laurich. L. Men (CREAIT Bio-imaging cluster, MUN) provided assistance with Scanning Electron Microscopy. Finally, I'd like to thank friends and family who encouraged and supported me from start to finish.

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Introduction

Ecological importance of thyasirid clams

Natural and anthropogenic factors are increasing the proportion of coastal marine sediments affected by organic enrichment worldwide (Rosenberg, 1985, Diaz & Rosenberg, 2001) through seasonal phytoplankton blooms, river runoff, aquaculture pens, hydrocarbon development, and sewage discharge. Organic enrichment often results in oxygen depletion and accumulation of sulphides in the sediment, conditions that are detrimental to most aerobic, sulphide-intolerant organisms (Dando *et al.*, 2004). Among the few macrofaunal organisms known to tolerate these conditions, marine thyasirid clams with symbiotic chemoautotrophic bacteria often occur in high abundance in environments rich in organic carbon, where they can serve as key indicator species (Pearson & Rosenberg, 1978; López-Jamar *et al.*, 1995). Symbiotic thyasirids obtain sulphides from the sediment (Dufour & Felbeck, 2003) and transfer them to chemoautotrophic sulphur-oxidizing bacteria associated with epithelial cells in their gills (Distel, 1998). Thyasirid symbionts use reduced sulphur as an energy source for carbon fixation (Spiro *et al.*, 1986), and provide nutrition for the clam (Dando *et al.*, 1986). Thyasirids have been referred to as "ecological engineers" (sensu, Jones *et al.*, 1994) because their activities can deplete sulphides in sediments, thereby improving conditions for other infauna (Dando *et al.*, 2004).

Symbiotic thyasirids are excellent models for understanding the function of organisms in organically enriched environments; such knowledge is invaluable for the

development of techniques related to environmental monitoring and remediation. Additionally, thyasirids are among the few bivalve families possessing both symbiotic and asymbiotic species (Dufour, 2005), with nutritional dependence on symbionts varying among species (Dando & Spiro, 1993; Dufour & Felbeck, 2006). These attributes make this group important for studying evolutionary aspects of host-symbiont co-evolution and speciation.

Thyasirids are found worldwide, from shallow to abyssal depths, making them a truly cosmopolitan group (Payne & Allen, 1991). Around the island of Newfoundland, Canada, thyasirids have been previously reported from several sites (e.g., Placentia Bay: Ramey & Snelgrove, 2003; and offshore oil fields: P. Pocklington, pers. comm.). Furthermore, thyasirids were collected from approximately 30 m depth in East Arm of Bonne Bay (Quijón & Snelgrove, 2005), and identified as *Thyasira flexuosa*, although they were never formally studied.

Thyasirid identification and taxonomy

Thyasirids are a taxonomically difficult group, due to the paucity of diagnostic characters (most associated with relative degrees of shell curvature) and the presumed high degree of variability in shell shape at the species level (Taylor *et al.*, 2007a). Assignment of a thyasirid to a particular species is problematic, and has led to frequent misidentifications, synonymies and very broad geographic distributions (Ockelmann, 1961; Oliver & Killeen, 2002). Thyasirid identification has been based mainly on shell characters, although features of the internal anatomy such as the number of gill

demibranchs, the morphology of the digestive diverticula and the shape of the foot can be useful traits for distinguishing between similar species (Payne & Allen, 1992; Oliver & Killeen, 2002). Gene sequencing can be particularly useful for species identifications in such a group, but has not yet been done extensively: most available thyasirid gene sequences in GenBank (of 18S and 28S rRNA, and COI) are from specimens from the Northeast Atlantic (e.g., United Kingdom, Norway); notably, there are none from the Northwest Atlantic. Gene sequencing can also inform on the dispersal potential of thyasirids. Many thyasirid species distributions are considered amphi-Atlantic (or pan-Arctic) - gene sequencing may help to confirm this assertion.

Recent studies investigating evolutionary relationships among thyasirids and other chemosymbiotic bivalves have been more focused on genetic than morphological characters (Williams *et al.*, 2004; Taylor & Glover, 2005; Williams & Ozawa, 2006). Such approaches revealed monophylogeny in the family Thyasiridae (Taylor *et al.*, 2007a) despite considerable morphological variation (Dufour, 2005). Molecular analyses also revealed that symbiosis likely evolved more than once within the Thyasiridae, with symbiotic species forming at least three distinct lineages (Taylor *et al.*, 2007a).

In Chapters 1 and 2, I characterize thyasirids from Bonne Bay using morphological traits (shell shape, gill filament appearance) and molecular markers (18S and 28S rRNA nuclear gene fragments), establishing the presence of an asymbiotic species, *Thyasira (Parathyasira)* sp, and a species complex affiliated with *Thyasira gouldi* (Chapters 1 & 2).

Host-symbiont specificity and co-speciation

Invertebrates with chemoautotrophic bacterial symbionts vary in their degree of symbiont specificity and host-symbiont co-speciation (Dubilier *et al.*, 2008). Distel *et al.* (1988) examined symbiont sequences from six marine invertebrates, three of which were bivalves from relatively shallow coastal environments, and found each host species had a unique symbiont phylotype (i.e., symbionts were host species-specific). In contrast, two species of co-occurring lucinid bivalves from a seagrass bed shared a symbiont phylotype (Durand *et al.*, 1996), and a host species of vestimentiferan tubeworm was associated with different symbionts at different sites (Vrijenhoek *et al.*, 2007). There is evidence for depth-specific adaptation and evolution in some bivalves, with a closer relationship between symbionts from a host species and symbionts of other invertebrate hosts from similar depths than to symbionts of congeners inhabiting different depths (Imhoff *et al.*, 2003). Therefore, certain host species appear to associate invariably with the same symbiont phylotype while others may associate with different symbionts, the latter possibly being acquired from the local environment. The mode of symbiont transmission (vertical: symbionts transferred between generations versus lateral: symbionts taken up from the environment or from co-occurring hosts) can be important in determining host-symbiont specificity (Dubilier *et al.*, 2008). When host species transmit their symbionts vertically, host and symbionts may co-speciate (Peek *et al.*, 1998; Goffredi *et al.*, 2003). Host species acquiring their symbionts laterally sometimes, but not always, show less symbiont specificity (e.g., lucinids, Gros *et al.*, 2003).

Symbionts of different thyasirid species are not closely related to each other, but are related to symbionts of other chemosymbiotic bivalves (Imhoff *et al.*, 2003). Because only six symbiont gene sequences are currently available, the host specificity of thyasirid symbionts is unknown and we lack evidence to support or refute co-evolution in this group. Thyasirid symbionts are thought to be acquired laterally, but the mechanism remains unverified. The research conducted here aims to determine whether closely related chemosymbiotic thyasirids from Bonne Bay possess unique symbiont phylotypes (i.e., host-specificity) or if different co-occurring thyasirid host phylotypes share symbionts (i.e., site specificity) (Chapter 3).

Nutritional dependence on symbionts

Chemosymbiotic hosts derive a proportion of their diet from their symbionts, through either direct translocation of products from symbiont to host, or through symbiont digestion (Le Pennec *et al.*, 1995; Distel, 1998; Le Pennec & Beninger, 2000). Thyasirids apparently acquire most nutrients from their extracellular symbionts through periodic endocytosis and digestion of these symbionts (Southward, 1986; Herry & Le Pennec, 1987; Dufour & Felbeck, 2006). Nutritional dependence of chemosymbiotic thyasirids on their symbionts varies among species (Dando & Spiro, 1993). Further, the nutritional dependence of thyasirids on their symbionts can vary according to ambient conditions: laboratory experiments showed that when particulate organic matter (POM) content in suspension was low, rates of symbiont digestion were high, and vice-versa (Dufour & Felbeck, 2006). Therefore, some thyasirid species can balance feeding modes

(suspension feeding or symbiont digestion) depending on environmental conditions, making them opportunistic species.

Other studies analyzed the stable carbon isotope ratios ($\delta^{13}C$) of thyasirid tissues to determine the proportion of host diet that is autotrophically-derived (more ^{13}C-depleted), versus heterotrophically-derived (more ^{13}C-enriched). In thyasirids, the temporal change in $\delta^{13}C$ depended on environmental conditions: $\delta^{13}C$ of thyasirids was similar to those of heterotrophic bivalves during periods of low sulphide availability, but were more negative (more ^{13}C-depleted) during periods of high sulphide availability, and suggested temporal changes in the relative importance of symbionts (Dando & Spiro, 1993). Similarly, stable isotope ratios of nitrogen and sulphide have been used to infer the relative importance of chemosymbiont-derived nutrition in invertebrates (Paull *et al.*, 1985; Brooks *et al.*, 1987; Kennicutt *et al.*, 1992; Vetter & Fry, 1998; Levin & Michener, 2002, Carlier *et al.*, 2010). Here, we determine the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{35}S$ of Bonne Bay thyasirids to evaluate the relative importance of chemoautotrophy to thyasirid nutrition at this site (Chapter 3).

Overview: Summary of Goals and Objectives

I studied the evolution of thyasirids from Bonne Bay by first characterizing the thyasirid species collected and comparing them to previously-described species from the Arctic and Eastern Atlantic (Chapters 1 & 2). Then, I studied the symbiont phylotypes of closely related thyasirid hosts to investigate host-symbiont specificity and co-speciation within Bonne Bay, and assessed the nutritional importance of symbionts in these thyasirids by determining stable isotope ratios (Chapter 3).

Co-Authorship Statement

Regarding the *design and identification of the research proposal*, my supervisor assisted with the initial research idea and gave constant feedback on the written proposal that I wrote and submitted after the first semester of my Masters. Regarding the *practical aspects of the research*, I conducted all of the work **except** preparing gill sections and taking images of these using light and transmission electron microscopes. Gill sections were prepared by Jason Laurich, who has been given co-authorship status because of these specific contributions. Regarding the *data analysis*, I fully conducted this work, receiving and applying advice given by my supervisor. Finally, I contributed fully to the *manuscript preparation*, writing each draft and incorporating the feedback given by my supervisor and committee.

Chapter 1: Characterization of a thyasirid species (Bivalvia: Thyasiridae) from a subarctic fjord in the Norwthwest Atlantic

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Abstract

The widely distributed Thyasiridae have recently attracted interest because of their association with sites affected by organic enrichment, and their potential role as ecosystem engineers that influence sediment reoxidation. Most research to date focused on thyasirids from the Eastern Atlantic, with little attention to the Western Atlantic. Using both morphological and molecular techniques, this study describes a thyasirid species from a subarctic fjord on the west coast of Newfoundland, Canada. Based on several key characteristics including shell morphology and a constructed 18S and 28S phylogeny, this species belongs to the subgenus *Parathyasira*. The Bonne Bay *Thyasira (Parathyasira)* species differs morphologically from the Arctic species *Thyasira (Parathyasira) dunbari*, and appears to be distinct from specimens of *Thyasira (Parathyasira) equalis* described from the east and deep Atlantic. Examination of the internal anatomy, and ideally, molecular sequences of *T. equalis* from the type location, the Gulf of Maine, is required to confirm whether the Bonne Bay species belongs to a western Atlantic subspecies of *T. equalis*, or to a new species. *Thyasira (Parathyasira)* sp. from Bonne Bay does not have chemoautotrophic bacterial symbionts.

Introduction

Thyasirid bivalves (superfamily Thyasiroidea, Taylor *et al.*, 2007a), originally described by Dall (1900), form a highly cosmopolitan group (Payne & Allen, 1991) inhabiting a wide range of marine environments from continental shelf and slope margins (Oliver & Killeen, 2002) to deep-sea cold seeps (Clarke, 1989; Fujikura *et al.*, 1999; Kamenev *et al.*, 2001; Oliver & Sellanes, 2005) and hydrothermal vents (Southward *et al.*, 2001). At higher latitudes, some thyasirid species inhabit and often dominate littoral sediments, fjords and estuaries affected by organic enrichment (hereafter OE) (Pearson & Rosenberg, 1978; Dando & Southward, 1986; Donval *et al.*, 1989; López-Jamar *et al.*, 1995; Oliver & Killeen, 2002; Oliver & Holmes, 2006; Wlodarska-Kowalczuk, 2007; Keuning & Schander, 2010; Keuning *et al.*, 2011). A renewed interest in the Thyasiridae, especially within the past decade, can be attributed to their association with OE industrial sites, including aquaculture pens, and in association with hydrocarbon production (Oliver & Killeen, 2002).

The thyasirid species found at OE sites often form a symbiotic relationship with chemolithoautotrophic bacteria (Southward, 1986; Dando & Southward, 1986; Keuning *et al.*, 2011) where the thyasirid provides a habitat and periodically digests symbionts, thus providing a source of nutrition in addition to that obtained through suspension feeding. The presence of lysosomal bodies and packed bacterial membrane whorls on transmission electron micrographs of bacteriocytes indicates symbiont digestion (Southward, 1986; Herry & Le Pennec, 1987; Dufour & Felbeck, 2006) whereas

histoautoradiographic studies (Southward, 1986) and the depleted ^{13}C signature of the clams' tissues demonstrates nutritional dependence of thyasirids on their symbionts (Spiro *et al.*, 1986; Dando & Spiro, 1993; Rodrigues & Duperron, 2011). Thyasirids use their foot to construct deep burrow networks beneath the shell to access reduced sulphur (Dufour & Felbeck, 2003) which they make available to their symbionts for use in the chemosynthetic pathway (Distel, 1998). Some thyasirids qualify as "ecosystem engineers", because they are thought to facilitate the recolonization of a greater diversity of benthic fauna (Dando *et al.*, 2004) by oxidizing the near burrow sedimentary environment.

Regardless of their presumed ecological importance, many gaps exist in our knowledge of thyasirids, especially from an evolutionary perspective. The thyasirid fossil record extends back to at least the Cretaceous (Kauffman, 1969; Taylor *et al.*, 2007a); pre-Cretaceous records are poorly understood most likely because of the expected small size of the shells, resulting in poorly preserved fossils (Taylor *et al.,* 2007a). Over 90 living species of thyasirids have been described to date, comprising around 11 genera (Coan *et al.*, 2000). However, the lack of key diagnostic morphological traits and the high intraspecific variability associated with the shell confound confident identification of thyasirid species using morphology alone, often leading to erroneous classification. For example, three distinct species, *Thyasira gouldi*, *Thyasira sarsi*, and *Parathyasira equalis* have been misidentified as *T. flexuosa*, greatly overestimating the distribution of the latter species (Ockelmann, 1958; Oliver & Killeen, 2002).

More recent studies used gene sequences, including both 18S and 28S nuclear rRNA, to identify species confidently and build phylogenetic trees investigating the monophyly and evolutionary history of various groups (e.g., Adamkewicz *et al.*, 1997; Williams *et al.*, 2004; Taylor & Glover, 2006; Williams & Ozawa, 2006; Taylor *et al.*, 2007b). For example, Thyasiridae were originally thought to be closely related to the Lucinidae, based on similarities in morphology, internal anatomy, and life habits that are related to the groups' association with chemoautotrophic sulphur-oxidizing bacteria; these two families were therefore placed in the superfamily Lucinoidea (Taylor *et al.,* 2007a). After molecular analysis of both 18S and 28S rRNA genes, however, Taylor *et al.* (2007a) found that the Thyasiridae form a highly supported monophyletic clade that occupies a near basal position in the heterodont bivalve phylogenetic tree, distinct from all other heterodonts including the Lucinidae. As prescribed by Taylor *et al.* (2007a), the classification and characterization of thyasirids should encompass a wide range of methods, including morphology (shell and internal anatomy) and molecular techniques.

Although Northeast Atlantic species, including *Thyasira gouldi* and *Thyasira flexuosa*, have been studied extensively, uncertainty persists whether these species exhibit amphi-Atlantic distributions; knowledge of which species occur in the Northwest Atlantic would contribute to a better understanding of the dispersal potential and gene flow of thyasirids. Determination of gill morphology and symbiont presence in thyasirid species can provide important insights on evolutionary processes in the family. For example, Dufour (2005) discovered that nine of the 26 thyasirid species studied harboured symbionts, and that symbiont presence could be related to gill filament morphology;

individuals possessing simple gills (type 1 or 2) were most likely asymbiotic or partially so, while all individuals possessing abfrontally expanded, 'type 3' gills possessed symbionts (Dufour, 2005). Further, the concatenated 18S and 28S tree constructed by Taylor *et al.* (2007a) showed that the symbiotic species studied occupied more basal positions than a highly supported clade of asymbiotic species, suggesting the latter may be more derived.

Here, we characterize a thyasirid species collected from OE sediments in Bonne Bay, a subarctic fjord in western Newfoundland, Canada. We base our characterization on morphological traits including key shell features as described in Oliver & Killeen (2002), larval shell diameter, internal anatomy, gill morphology, and symbiont presence. Molecular analysis of both 18S and 28S nuclear rRNA gene fragments further support these morphological observations.

Materials and Methods

Study site

Bonne Bay, a subarctic fjord system located on the west coast of Newfoundland, Canada (49^{0}30'N 57^{0}55'W, **Figure 1-1**: A), is partially separated from the Gulf of St. Lawrence by a sill (~ 50 m depth). The fjord is comprised of two "arms", South Arm (55 m maximum depth) and East Arm (230 m, maximum depth) (Quijón & Snelgrove, 2005), the latter further separated by a shallow sill (~15 m depth) (**Figure 1-1**: B). The partial separation from the open ocean and riverine input result in fluctuating salinities in some

of the more shallow areas (< 15m) of East Arm based on salinity probe measurements conducted in this study (salinity range: 28.6 - 30.1), whereas deeper areas (100-200 m) are more stable, with temperatures ranging from -1 to +1 ºC (Comeau *et al.,* 1991; Gilbert & Pettigrew, 1993). Organic enrichment from river run-off into East Arm carries terrestrial organic matter inputs that become confined in the narrow channels of the bay (R. Hooper, pers. comm.). Although several locations in the Bonne Bay fjord were initially sampled, including South Arm, thyasirids were only found at three East Arm collection sites: Southeast Arm ("S", 49°27'51.46"N, 57°43'09.04"W, 30 m depth), Deer Arm ("D", 49°32'43.48"N, 57°50'28.45"W, 30 m depth), and Neddy's Harbour ("N", 49°31'21.44"N, 57°52'11.07"W, 15 m depth). Sites D and N are separated by approximately 4 km, sites D and S by 14 km, and sites S and N by 13 km.

Thyasirid collection

The specimens used in this study were collected on three separate occasions: October 2009, May 2010, and August 2010. We collected sediments with a Peterson grab (radius = 10.5 cm, height = 30 cm, volume ~ 2.08×10^4 cm^2), sieving out thyasirids on a 1 mm mesh. We then identified and quantified thyasirids from these sites roughly to species based on the key shell characters described in Oliver & Killeen (2002). Although we identified more than one thyasirid species during these sampling trips, we focus here on only one species.

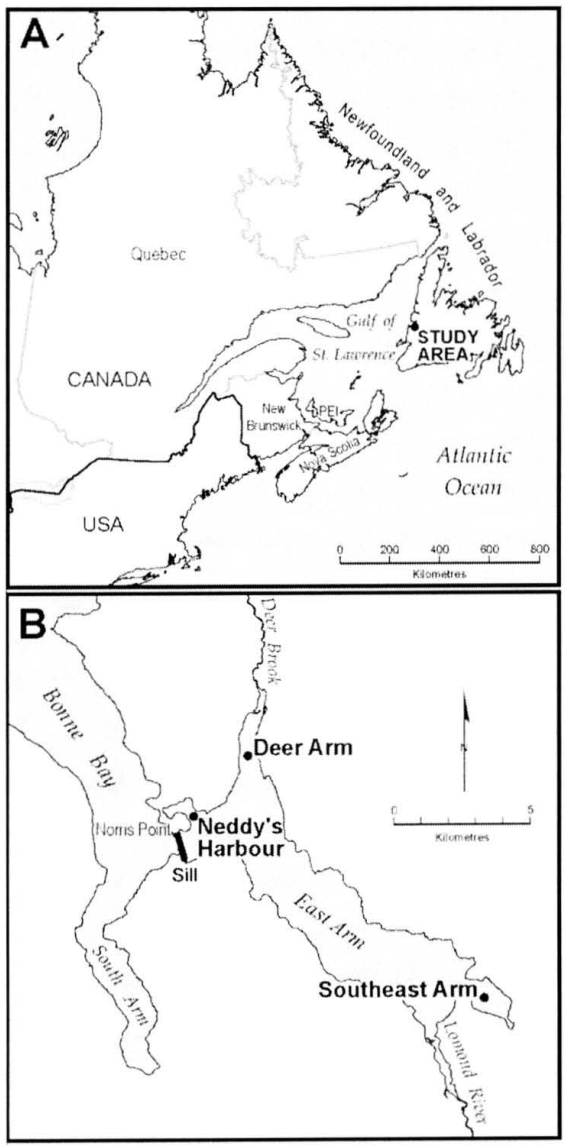

Figure 1-1: Map of study site and surrounding area. A. Study area (closed dot) in Newfoundland, Canada. B. Study area (Bonne Bay) with sites indicated: Southeast Arm, Neddy's Harbour, and Deer Arm.

Environmental parameters and abundance

Given the nutritional dependence of symbiotic thyasirids on sulphides (Dando & Southward, 1986), and because many thyasirids inhabit OE sediments, we expected free sulphide concentrations and percent organic matter would affect thyasirid distributions. To test these effects, we obtained three sediment samples using 5 mm diameter tube cores from every grab containing thyasirids, and immediately placed samples into sealed microcentrifuge tubes. Upon arrival at the laboratory (normally within 1 h of collection), we placed tubes in a nitrogen-filled glove bag, where we took further steps (porewater extraction by centrifugation, and pipetting of porewater into traps containing 4 parts 2.6% zinc acetate to 1 part 6% NaOH) to minimize sulphide loss. Determinations of free sulphide concentrations were performed based on the spectrophotometry protocol of Gilboa-Garber (1970). After porewater extraction, the remaining sediments were placed in a drying oven at 60 °C, left to dry overnight, and then weighed to obtain the dry weight of each sample. Percent organic matter was calculated using standard percent loss on ignition, ash weight determined after placing dried samples into a muffle oven at 500 ^{0}C and left for 3 hours.

Thyasirid abundance was calculated as the sum of individuals at a given site, regardless of sampling period. We used the statistical analysis program JMP (v. 10, SAS Institute Inc., Cary, NC, USA) to determine whether sites (D, N, S) significantly differed in free sulphide concentrations (μM) and organic matter (%) using standard least squares models. Both sets of data (sulphide and OM) were log transformed in order to meet assumptions on residuals. We also conducted two Tukey's HSD multiple comparisons

tests for both sulphide and OM% and constructed bar charts with confidence intervals to visualize parameter differences between sites.

Scanning Electron Microscopy of shells

To obtain quality shell images, as well as measurements of the prodissoconch for species diagnosis, we placed six shell valves in an ultrasonic water-filled bath to remove debris from their surface. Cleaned valves were mounted on stubs and sputter-coated with gold for observation with a Hitachi S-570 Scanning Electron Microscope, or directly observed without gold coating with an FEI Quanta 400 SEM under variable pressure mode. We measured larval shell diameter using the computer software ImageJ (Abràmoff *et al.*, 2004).

DNA extraction, PCR amplification and sequencing

The gills of eight individuals (including five specimens from which one gill was retained for microscopy, as described below) were immediately placed in 95% ethanol following dissection, and treated as single DNA samples (i.e., no tissues from different individuals were pooled). DNA was isolated from the gill tissue and purified using the QIAgen DNeasy® Blood and Tissue kit, following the spin-column protocol for animal tissues. Polymerase chain reactions (PCRs) used two sets of primers: 18S5' (Winnepenninckx *et al.*, 1998) and 18S1100R (Williams & Reid, 2004), as well as LSU5' (Littlewood *et al.*, 2000) and LSU1600R (Williams *et al.*, 2003) for thyasirid 18S and 28S rRNA genes, respectively. Because most studies of thyasirids and other molluscs used

both of these highly conserved nuclear genes (ex. Adamkewitcz *et al.*, 1997; Williams *et al.,* 2003; Williams & Reid, 2004; Williams *et al.,* 2004, Williams & Ozawa, 2006; Taylor *et al.,* 2007a, Taylor *et al.,* 2007b, Distel *et al.,* 2011), we could compare the sequences obtained from this study to a larger data set. Combined (i.e., concatenated) use of 18S and 28S increases the amount of base positions (bp) in the phylogenetic analysis (~2500 bp in total), further increasing the statistical confidence associated with tree branch support values (see Taylor *et al.*, 2007a).

We performed each 25 µL PCR reaction using the Promega PCR Master Mix (Promega Corp.) containing 50 µ/mL Taq DNA polymerase, 400 µM of each dNTPs, 3mM $MgCl_2$, reaction buffer at a pH of 8.5, and adjusted according to optimal primer conditions (see **Table 1-1** for summary). We set thermocycler settings to: initial denaturation at 94 ^{0}C for 4 min, 35 cycles of denaturation at 94 ^{0}C for 30 sec, a primer-specific annealing temperature of 54 ^{0}C for 18S and 52 ^{0}C for 28S, and elongation at 72 ^{0}C for 2 min, with a final elongation at 72 ^{0}C for 5 min. All amplified products were filtered using Acro-Pro® 100K-Omega filters (Pall Life Sciences). After filtration, we performed sequencing reactions using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems) and electrophoresed samples on an Applied Biosystems 3730 DNA Analyzer automated capillary sequencer using Sequencing Analysis v. 5.2 Software (Applied Biosystems).

Table 1-1: Forward and reverse primer pairs and the primer-specific conditions used.

Target DNA	Name	Sequence (5'-3')	Annealing Temp (°C)	$MgCl_2$ (mM)	Source
18S	18S-5'	CTGGTTGATYCTGCCAGT	54	3	1
	18S1100R	CTTCGAACCTCTGACTTTCG			2
28S	LSU5(F)	TAGGTCGACCCGCTGAAYTTAAGCA	52	2.5	2,3
	LSU1600R	AGCGCCATCCATTTTCAGG			

1. Winnepenninckx *et al.*, 1998; 2. Williams *et al.*, 2003; 3. Littlewood *et al.*, 2000

Phylogenetic analyses

We aligned and compared both the forward and reverse sequences using Sequencher® (v. 4.8, Gene Codes Corp.) in order to verify bases, and then matched edited sequences with the most closely related species using the online Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990). We next aligned our thyasirid sequences with several other previously published thyasirid sequences (**Table 1-2**) using ClustalW (Thompson *et al.*, 1994). All alignments were executed with default values in MEGA5 (Tamura *et al.,* 2011). To improve alignments, we used the software Gblocks (0.91b, Castresana, 2000), the final alignment containing 986 bp (96%) of the original 18S alignment and 1427 bp (96%) of the original 28S alignment.

Phylogenetic reconstruction

We constructed a Bayesian tree on the concatenated 18S and 28S alignment using MrBayes (v 3.1.2, Huelsenbeck & Ronquist, 2001). The two best models for estimating these genes were the Kimura 2-parameter (Kimura, 1980) for 18S and Tamura-Nei (1993) for 28S, based on Bayesian Information Criterion scores calculated using the Maximum-Likelihood statistical method (Nei & Kumar, 2000) in MEGA5. The concatenated tree partitioned 18S and 28S fragments, applying the chosen gene-specific models. For both genes, we estimated base frequencies and set rate variation to gamma-distribution with four discrete categories and allowance for invariant sites. We ran data sets twice, with 500,000 generations each and a sampling frequency of once every 100 generations, basing the consensus tree on the combination of the remaining trees from each run (7500

trees in total). Support values associated with the nodes of the tree were based on posterior probabilities calculated in MrBayes.

Light and Transmission Electron Microscopy of gills

Upon collection, we dissected and removed thyasirid (N = 17) gills, retaining one gill for five of those individuals for morphological analysis, and the other for DNA extraction, as described previously. Gills were fixed in 2.5 % gluteraldehyde in 0.1 M sodium cacodylate buffer for 24 hours before transfer to a 0.1 M sodium cacodylate buffer. We then stained gill samples with 1% osmium tetroxide in the same buffer, and dehydrated in an ascending ethanol series before embedding them in EPON resin. Embedded gills were sectioned using a LKG Bromma 8800 ultramicrotome, using a diamond knife to produce semi-thin (1 μm) sections, which we stained with 1% toluidine blue in 1% sodium borate for light microscopy. We also made ultra-thin sections (60 nm) using the same ultramicrotome, which we observed using a Philips 300 transmission electron microscope after post-staining sections with uranyl acetate and lead citrate.

Table 1-2: Thyasirid and other bivalve species included in the 18S+28S concatenated tree, with collection locality and GenBank accession numbers given for each gene fragment used.

Species	Locality	18S rRNA	28S rRNA
Thyasira sarsi	UK: Northern North Sea	AM774485	AM779659
Parathyasira equalis	Sweden: Gullmarsfjord	AM774482	AM779656
Mendicula ferruginosa	UK: Northern North Sea	AM774483	AM779657
Adontorhina cyclia	USA: San Diego	AM392455	AM392438
Axinulus sp.	Antarctica: Scotia Ridge	AM392441	AM392440
Thyasira methanophila	Chile: Concepcion	AM392447	AM392431
Leptaxinus indusarium	Pakistan: Arabian Sea	AM392454	NA
Thyasira perplicata	Angola	AM392448	AM392432
Thyasira polygona	UK: Northern North Sea	AM774484	AM779658
Thyasira flexuosa	UK: Plymouth	AJ581870	AJ581903
Thyasira gouldi	UK: Mill Bay, Salcombe	JF899224	JF899196
Thyasira cf. subovata	Antarctica:Scotia Ridge	AM392451	AM392435
Carditamera floridana	USA: Manatee Co.	AF229617	NA
Eucrassatella cumingii	Australia: Moreton Bay	AM774479	AM779653

Results

Morphological characters

The species characterized here (size range = 3-6 mm; N = 17) belongs to the subgenus *Parathyasira* (hereafter *Thyasira (Parathyasira)* sp.), based on the following shell characteristics: an absent auricle, and a well-defined submarginal sulcus with a sunken escutcheon, along with a poorly defined, truncate posterior area (Oliver & Killeen, 2002) (**Figure 1-2:** A & B). Shells are transparent, thin, and moderately inflated, with a subequilateral - rhomboidal outline (sensu, Oliver & Killeen, 2002). Beaks are slightly anterior to the midline, length slightly exceeds height, and anterior, posterior and ventral margins are acute. The lunule margin is short, with a smooth, not granulate shell surface, and no evident muscle scars. The prodissoconch measured on average 138 µm (size range: 122-156 µm; N = 6), and lacked radial folds (**Figure 1-2**: C).

The internal anatomy of Bonne Bay specimens was similar among observed individuals. The lateral pouches (containing digestive diverticula) in most individuals were dark brown, with large, distinctly-shaped and separate lobes (**Figure 1-2**: D); we frequently observed white oval eggs measuring approximately 80 µm on the inner surface of the pouches. Gills appeared translucent in most specimens, and consisted of two demibranchs, the inner about a third the size of the outer demibranch. The foot was vermiform and elongate, with a well-defined tip.

To improve our identification of the Bonne Bay *Thyasira (Parathyasira)* species, we examined preserved *T. (Parathyasira) dunbari* material collected from Canadian

ArcticNet expeditions to the Beaufort Sea in 2009 and 2010. Specimens were collected by box core from approximately 600 m depth, and fixed in buffered formaldehyde. The shells of all thyasirids examined from the Beaufort Sea (N = 45, size range: 2-4 mm) had a pronounced submarginal sulcus, a subequilateral – rhomboidal outline, and no auricle (**Figure 1-2**: E). The internal anatomy of *T. dunbari* from the Beaufort Sea was similar to that of the Bonne Bay specimens, apart from less well-defined lobes of digestive diverticula that occupied a relatively larger volume of the mantle cavity (**Figure 1-2**: F).

Environmental parameters and abundance

The vast majority of thyasirids from this study and additional sampling trips were found at Southeast Arm (N=92), although they were occasionally found at Deer Arm (N =19); they were never observed at Neddy's Harbour. Based on the Tukey's HSD multiple comparisons test for free sulphide concentrations, we found significantly higher concentrations at site S (x=21.8, SE=0.1) than site D (x=14.0, SE=0.1). Neddy's Harbour (x=20.5, SE=0.1) was not significantly different compared to either S or D (**Figure 1-3**: A). For organic matter percentages, we found significantly higher proportions for sites S (x=12.0, SE=0.3) and D (x=11.4, SE=0.3) compared to Neddy's Harbour (x=3.5, SE=0.3) (**Figure 1-3**: B).

Figure 1-4: Morphology of *Thyasira (Parathyasira)* sp. collected from Bonne Bay, Newfoundland, and of *Thyasira (Parathyasira) dunbari* from the Beaufort Sea. A. Outer view of the left valve of *T. (Parathyasira)* sp., showing a flattened and poorly defined posterior area (pa), a rounded posterior margin, and no auricle. B. Inner view of the right valve of the same species. C. Scanning electron micrograph of the larval shell of *T. (Parathyasira)* sp. (~136 μm in diameter). D. Internal anatomy of *T. (Parathyasira)* sp., revealing the lobate digestive ceca (dc), foot (f), gill (g) with two demibranchs, and mantle margin (m). E. Outer view of the left valve of *Thyasira (Parathyasira) dunbari*. F. Internal anatomy of *Thyasira (Parathyasira) dunbari*. dc: digestive ceca, f: foot, g: gill, m: mantle margin.

Molecular identification

For the eight individuals used in the genetic analyses, we observed no base discrepancies in either the 18S or 28S gene fragments. The 18S sequence most closely matched both *Thyasira sarsi* (AM774485) and *Thyasira* (*Parathyasira*) *equalis* collected from the Gullmarsfjord, Sweden (AM774482) in the BLAST search, each with a 98% sequence similarity. For the 28S consensus sequence, the closest match was *Axinulus* sp. (AM392440), with a lower (96%) sequence similarity, followed by *T. (Parathyasira) equalis* (AM779656) with a 95% similarity. The absence of *T. (Parathyasira) dunbari* gene sequences in GenBank is noteworthy, and formalin preservation precluded genetic analysis of specimens from the Beaufort Sea.

Phylogeny

The phylogenetic reconstruction based on concatenated 18S and 28S sequences (Figure 1-4) revealed that *Thyasira (Parathyasira)* sp. from Bonne Bay is most closely related to *T. sarsi* and *T. methanophila*, occupying the same clade with a high support value (97%). *Thyasira (Parathyasira)* sp. was excluded from the clade containing asymbiotic thyasirids possessing only one demibranch (*Mendicula ferruginosa*, *Adontorhina cyclia*, *Axinulus* sp. and *Leptaxinus indusarium*) and, in contrast to the BLAST results based on percent similarity, was not closely related to *Thyasira (Parathyasira) equalis* from the Gullmarsfjord.

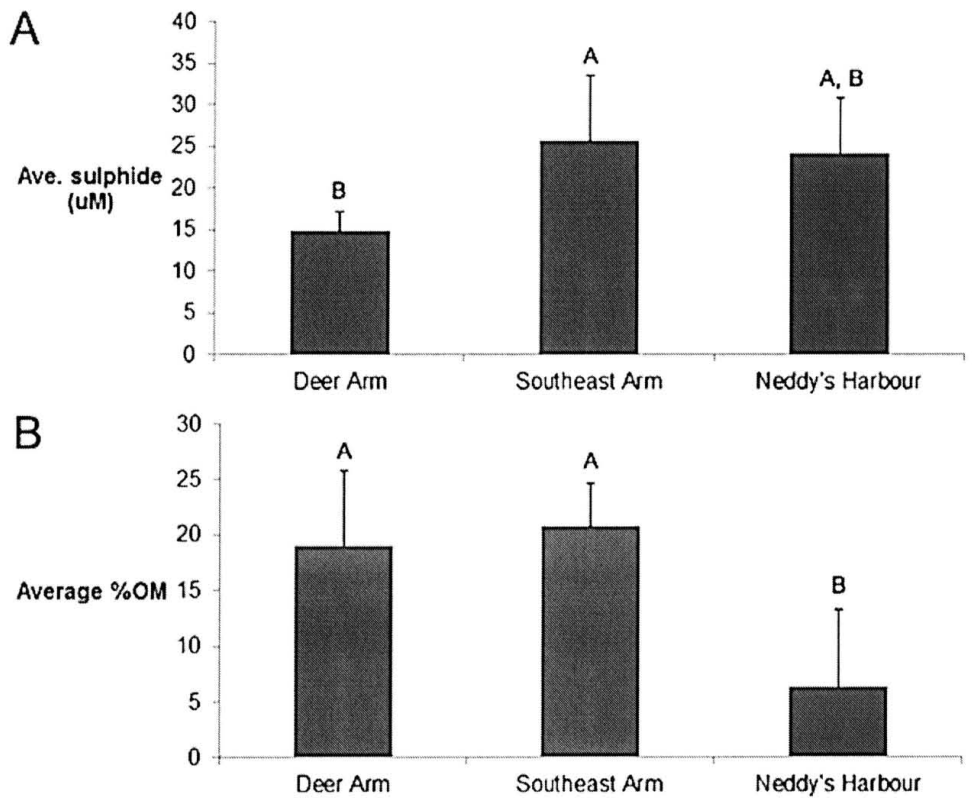

Figure 1-3: Bar charts with 95% confidence intervals indicated for each site showing averages. A. free sulphide concentrations in sediment porewater, and B. sediment organic matter percentages. Bars not connected with same letter (A,B) are significantly different, based on Tukey's HSD multiple comparisons test.

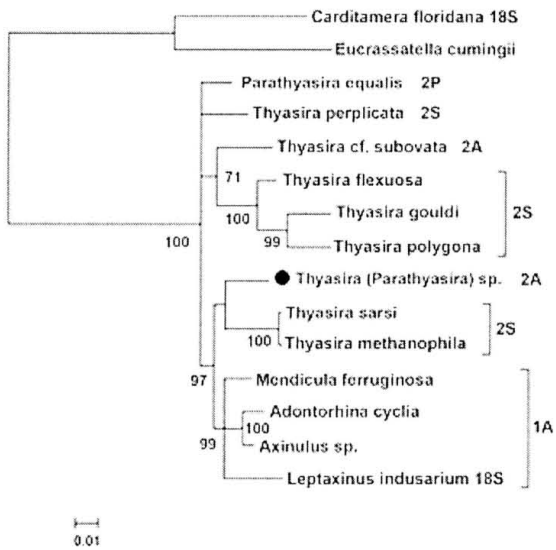

Figure 1-4: Concatenated 18S + 28S Bayesian tree showing phylogeny of thyasirids collected from Bonne Bay (λ) , and other species from various localities (see Table 1-2 for accession numbers). All species had both 18S and 28S sequences available, unless indicated (18S only, included for completeness). Outgroup composed of species *Carditamera floridana* and *Eucrassatella cumingii*. Symbiotic (S), asymbiotic (A), and partially symbiotic (P) species, as well as number of gill demibranchs (1 or 2) indicated next to species names. Support values calculated as posterior probabilities shown next to branches, unless < 50%, in which branches are collapsed. Branch lengths in units of # base substitutions per site.

Gill Structure and ultrastructure

We retrieved all of the examined specimens (N = 17) from site S, and confirmed the identity of five of those specimens by gene sequencing. In transverse section, gill filaments were of the kind described by Dufour (2005) as 'type 2', with simple filaments characterized by little or no abfrontal expansion throughout their dorso-ventral length (**Figure 1-5**: A). The cellular organization of this region was consistent among individuals. In the abfrontal zone of *Thyasira (Parathyasira)* sp. gill filaments (i.e., abfrontal to the lateral ciliated cells), the epithelium was pseudostratified, with three types of epithelial cells. Enlarged mitochondria containing limited cristae, many of which appear as small sacs, filled the largest and most conspicuous cells (c1, **Figure 1-5**: B-E). These cells were basal to another cell type, which formed a thin pseudolayer covering the apical surface of the cells with enlarged mitochondria (c2, **Figure 1-5**: B, D, E). C2 cells contained apically-located nuclei, mitochondria smaller than in c1 cells, membrane-bound organelles resembling lysosomes, and an apical surface bearing microvilli (**Figure 1-5**: B & D). A third cell type in the abfrontal area consisted of mucocytes, which were stained in pink with toluidine blue (**Figure 1-5**: C), and commonly occurred near the abfrontal end of filaments.

This species lacks bacterial symbionts, as evidenced by the absence of bacteria in the cytoplasm, or among the microvilli, of epithelial cells. In one individual, a few bacterial cells were observed in the cytoplasm of a c2 cell located adjacent to a lateral ciliated cell (**Figure 1-5**: E & inset).

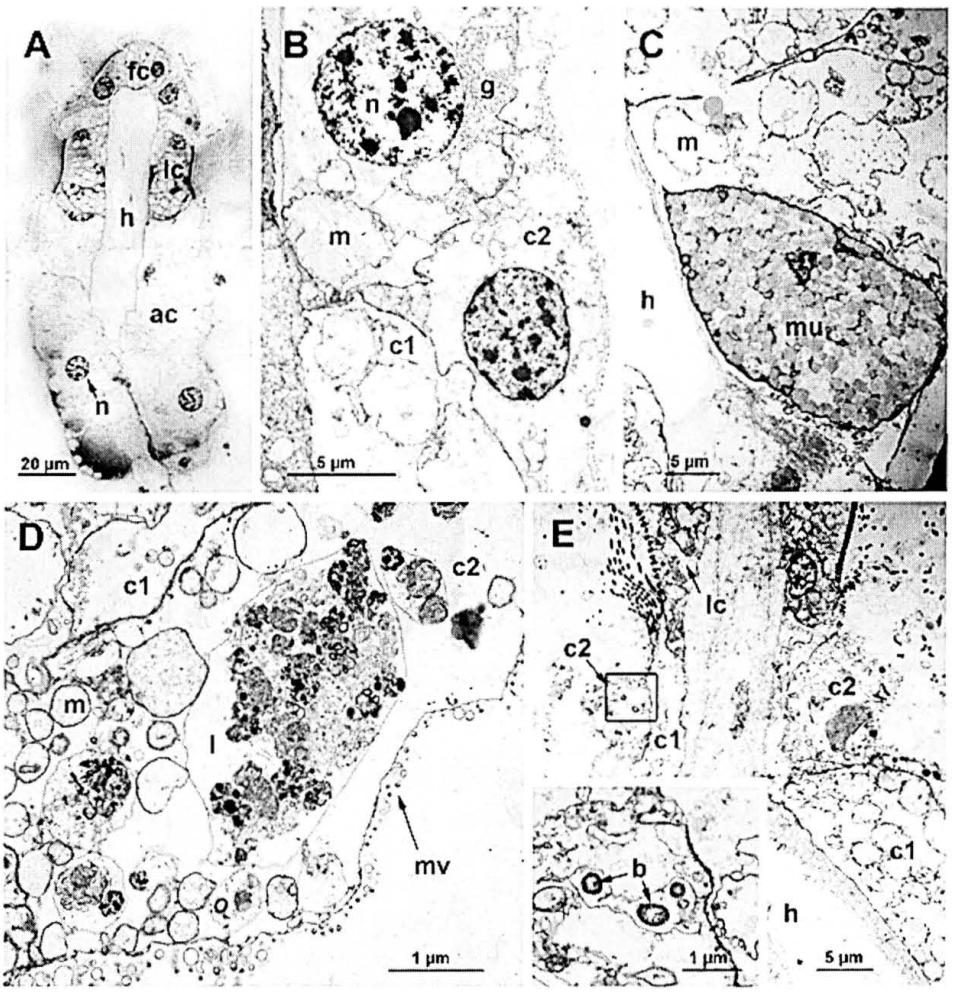

Figure 1-5: Light micrograph (A) and transmission electron micrographs (B - E) of the gills of *Thyasira (Parathyasira)* sp. A. Transverse section of a gill filament, showing frontal ciliated zone and abfrontal cells. Note uneven staining in abfrontal-most cell. B. Pseudostratified epithelium in the abfrontal zone, with c1 basal to c2. Accumulation of glycogen corresponds to greater intensity of staining with toluidine blue (**Figure 1-5**: A). C. Mucocyte in the abfrontal zone. D. Apparent lysosome in a c2. E. Bacteria (at higher magnification in inset) in c2. Box shows location of inset. Abbreviations: ac, abfrontal cell; b, bacteria; c1, basal-most abfrontal cell; c2, apical-most abfrontal cell; fc, frontal ciliated cell; g, glycogen; h, hemocoel; l, lysosome; lc, lateral ciliated cell; m, mitochondrion; mu, mucocyte; mv, microvilli; n, nucleus.

We successfully amplified the universal bacterial 16S gene (Weisburg *et al.,* 1991) in three of eight *Thyasira (Parathyasira)* sp. gills studied, including the specimen shown in **Figure 1-5**: E. Sequences from two individuals were identical, and the third was highly similar; these sequences bore 97% similarity to an uncultured deltaproteobacterium obtained from Arctic sediment (accession number EU050850).

Discussion

The species of the subgenus *Parathyasira* collected from Bonne Bay shares some similarities with both *Thyasira (Parathyasira) dunbari* (Lubinsky, 1976) and *Thyasira (Parathyasira) equalis* (Verrill & Bush, 1898). This similarity is hardly surprising given that past studies confused *T. (Parathyasira) dunbari* for *T. (Parathyasira) equalis* (Oliver & Killeen, 2002).

T. (Parathyasira) dunbari is considered a high Arctic species from the Canadian-Greenlandic region (Oliver & Killeen, 2002); although Bonne Bay is farther south than this region, numerous Arctic species occur in Bonne Bay, likely as a result of larval transport south from the Labrador Current, and stable conditions in the deeper, colder water layer of this fjord. *T. (Parathyasira) dunbari,* a shallow water (10-70 m depth) species, inhabits fjords and bays composed of muddy sediment, usually near river outwash fans (Lubinsky, 1976). Collections from the Beaufort Sea indicate that this species can also occur in greater depths (to approximately 600 m). In Bonne Bay, we collected *T. (Parathyasira)* sp. from sites S and D, at around 30 m depth, on muddy

bottoms near the outwash fans of rivers (Deer Brook in D and Southeast Brook in S). We could not find individuals at the shallower site N, characterized by more variable temperatures and salinities (data not shown), coarser sediments, and significantly less organic matter. *T. (Parathyasira) dunbari* was recently reported as a dominant species in a glacial bay of Kongsfjorden, on the west coast of Spitzbergen (Wlodarska-Kowalczuk, 2007). Similarly to Bonne Bay, this fjord has subarctic characteristics because of warm Atlantic water inflow from the West Spitsbergen current, as well as relatively low- to-moderate organic content in sediments (0.1–0.2 mg/g), a high degree of sedimentation, and a high proportion of silt-rich sediments (Fetzer *et al.*, 2002; Wlodarska-Kowalczuk & Pearson, 2004; Wlodarska-Kowalczuk, 2007). Silt concentration, but not organic matter, strongly predicted *T. (Parathyasira) dunbari* distribution in Kongsfjorden (Wlodarska-Kowalczuk, 2007); the same could be true of *T. (Parathyasira)* sp, explaining its absence from Neddy's Harbour.

Thyasira (Parathyasira) equalis, in contrast, spans a greater depth range, with specimens collected at coastal depths off Scandinavia, Greenland and northeast America (from Nova Scotia to Chesapeake Bay), to depths of up to 4734 m in the Atlantic Ocean (Payne & Allen, 1991). It was conspicuously absent, however, in oil field samples (Oliver & Killeen, 2002) expected to be relatively high in organic matter. *T. equalis* was reported at shallower depths (27 m), in coastal waters off of Western Norway, where sediment organic matter ranged from 2.7 to 33.3% (Keuning *et al.*, 2011). However, this species is expected to thrive in a less organically enriched environment (Keuning *et al.,* 2011), similarly to *T. dunbari* as described in Wlodarska-Kowalczuk (2007). Although genetic

evidence on the occurrence of *T. equalis* and *T. dunbari* off Newfoundland has not yet been reported, both species could potentially inhabit depths and sediments similar to those in the Bonne Bay sites based on their expected distributions.

The shell of *Thyasira (Parathyasira)* sp. from Bonne Bay differs from that of *T. (Parathyasira) dunbari* from the Beaufort Sea in: a) a truncated, rather than a rounded posterior margin; 2) a shorter submarginal sulcus; 3) the lack of a cardinal tooth; 4) beaks located closer to the midline; and 5) a less anteriorly-elongated outline (with length exceeding height). Although Lubinsky (1976) noted that smaller individuals were more rounded than pyriform, the shells of individuals compared here (**Figure 1-2**: A & E) were roughly of the same size, so the difference in outline is unlikely growth-related. However, Ockelmann (1958) observed that *T. dunbari* from East Greenland are more rounded in outline than specimens from the high Arctic (Schiøtte, 1989). Compared to the shell of *T. (Parathyasira) equalis* (Payne & Allen, 1991; Oliver & Killeen, 2002), that of the Bonne Bay *Parathyasira* species lacks radial folds on the prodissoconch, and is longer than high (including in larger individuals) instead of the opposite. Prodissoconch size was not useful for distinguishing between *T. equalis* and *T. dunbari* since their size ranges overlap: Lubinsky (1976) reports a size of 160 μm for *T. dunbari*, whereas Oliver & Killeen (2002) note a generally larger prodissoconch in *T. dunbari* than in *T. equalis* (range: 155-167 μm); in both cases, reported sizes exceed those of Bonne Bay *T. (Parathyasira)* sp. Latitudinal gradients in prodissoconch size reported in *T. gouldi* (Oliver & Killeen, 2002) may reflect a common trend in thyasirids, so prodissoconch size should be used cautiously in species diagnostics.

Examination of the internal anatomy revealed differences between the Bonne Bay *Thyasira (Parathyasira)* sp., *T. (Parathyasira) equalis* and *T. (Parathyasira) dunbari*. Anatomical characters have been described for *T. (Parathyasira) equalis* collected from the eastern Atlantic (Oliver & Killeen, 2002); type material from the Gulf of Maine was described as bearing strong similarities with specimens from the eastern Atlantic (Oliver & Killeen, 2002), and specimens collected from the deep Atlantic were illustrated by Payne & Allen (1991). Descriptions of the internal anatomy of *T. (Parathyasira) dunbari* from the Canadian Arctic Archipelago are limited: Oliver & Killeen (2002) mention Ockelmann's personal observation that "the anatomy is similar to that of *T. equalis*". Based on the new observations we report for the Beaufort Sea material, the main characteristic that distinguished the Bonne Bay species from *T. (Parathyasira) dunbari* and *T. (Parathyasira) equalis* from the eastern Atlantic is the form of the digestive diverticula, which is more lobate and digitate in the Bonne Bay specimens. Illustrations of a *T. (Parathyasira) equalis* from 800 m depth in the North America Basin are more reminiscent of the Bonne Bay material, particularly in the form of the digestive diverticula (Payne & Allen, 1991).

The concatenated phylogenetic tree (**Figure 1-4**) revealed that *Thyasira (Parathyasira)* sp. was only distantly related to *Thyasira (Parathyasira) equalis* from the Gullmarsfjord, despite similarities in morphology and other characteristics. The *Parathyasira* clade may therefore not be monophyletic. *Thyasira (Parathyasira)* sp. was placed in a highly supported clade (97%) containing another highly supported clade (99%) of asymbiotic species with only one demibranch as well as symbiotic species with

two demibranchs. Nonetheless, the paucity of available thyasirid sequences leaves the phylogenetic placement of *Thyasira (Parathyasira)* sp. poorly resolved. The analysis of gene sequences of *T. (Parathyasira) dunbari* and western Atlantic *T. (Parathyasira) equalis* individuals would help in ascertaining the identity of the Bonne Bay species obtained here. Two sequences, *Carditamera floridana* and *Leptaxinus indusarium* had only 18S available, reducing the confidence of their position in the phylogeny. However, their presence in the phylogeny is not expected to affect the position of *T. Parathyasira* sp., as they were distantly related. Further, incomplete sequences have been used in other studies for the sake of completeness (Taylor *et al.*, 2007a), and were not expected to affect the credibility of the phylogeny.

T. (Parathyasira) sp. from Bonne Bay possess 'type 2' gills with a pseudostratified abfrontal epithelium and cells with enlarged mitochondria, as is typical of some thyasirids, such as *Thyasira (?) obsoleta* and *T. equalis* from the North Sea and the Barents Sea (Dufour, 2005). Most thyasirids with 'type 2' gills are asymbiotic; however, symbionts have been observed among the microvilli of c2 abfrontal cells in *Axinulus croulinensis* and in some, but not all, observed specimens of *T. equalis* (Dufour, 2005). In contrast, symbionts were absent in the 17 specimens of *T.* (*Parathyasira)* sp. we observed. Although we observed a few bacterial cells within the cytoplasm of a c2 cell, those are most likely pathogens or non-symbiotic bacteria engulfed by epithelial cells as a defense mechanism. The presence of lysosomes in another c2 cell could indicate widespread endocytosis and subsequent digestion of bacteria from the epithelial surface. The amplified deltaproteobacterial sequences likely belong to sediment-dwelling

sulphate reducers brought into the mantle cavity via the inhalant current and trapped in a mucociliary layer atop the gill epithelium, rather than to the bacterial cells seen in **Figure 1-5**: E. Although some bacterial phylotypes associated with bathymodiolid gills, and identified as symbionts, occur in low abundance (Duperron *et al.*, 2008), and deltaproteobacterial symbionts have been reported in marine sponges (Liu *et al.*, 2011) and oligochaetes (Ruehland *et al.*, 2008), the rarity with which we observed bacteria suggests it very unlikely they represent true symbionts.

The presence of enlarged mitochondria in abfrontal epithelial cells of *Thyasira (Parathyasira)* sp. gills is intriguing. Mitochondria in several animals play a role in sulphide oxidation and detoxification (Powell & Somero, 1986; Grieshaber & Volkel, 1998; Theissen & Martin, 2008), and vary in density and ultrastructure in different tissues or cellular layers (Dubilier *et al.,* 1997). Although relationships between mitochondrial ultrastructure and environmental sulphide or oxygen levels are not straightforward (Duffy & Tyler, 1984; Dubilier *et al.*, 1997), mitochondria with unusual features (swollen appearance, reduced cristae, and electron dense inclusions) are common in invertebrates from sulphide-rich environments, particularly in tissues exposed to sulphides (Menon & Arp, 1993; Janssen & Oeschger, 1992; Menon *et al.*, 2003). Swollen, electron lucent mitochondria with reduced cristae, resembling those observed here in *T. (Parathyasira)* sp., were reported in the epidermis of priapulids following 7 days of exposure to sulphide (Janssen & Oeschger, 1992). Whether these unusual mitochondria play a role in sulphur metabolism (Menon *et al.*, 2003), or signal mitochondrial injury as a result of sulphide exposure (Hance *et al.*, 2008) remains unclear. Typically, cellular stress is indicated by

the presence of unusual mitochondria and membrane-bound, electron dense organelles, interpreted as being mitochondria-degrading autophagosomes (Hance *et al.*, 2008). In *T. (Parathyasira)* sp., we observed putative lysosomes or autophagosomes in gill epithelial cells, but could not confirm their identity.

Conclusion

The *Thyasira (Parathyasira)* species collected from Bonne Bay, Newfoundland appears distinct from the Arctic species *T. (Parathyasira) dunbari*, based on differences in shell and anatomical characters. It also appears to differ from the eastern Atlantic *T. (Parathyasira) equalis*, based on anatomical characters, lack of symbionts, and gene sequences. However, it is possible that *T. (Parathyasira) equalis* from the eastern and western Atlantic are, in fact, different species. Additional observations of anatomical and symbiosis characters, as well as gene sequences of confirmed *T. (Parathyasira) equalis* from the type location (Gulf of Maine) could help resolve this question, and clarify whether the reported ranges of thyasirids are realistic. *T. (Parathyasira)* sp. from Bonne Bay inhabits sediments with high organic matter content, but lacks bacterial symbionts in its gill.

Chapter 2: Uncovering a species complex of thyasirids (Bivalvia: Thyasiroidea) in a subarctic fjord using both molecular and morphological analyses

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Abstract

The Thyasirid family is a model for early stages in the evolution of bivalve chemosymbiosis because it contains both asymbiotic and symbiotic species, and the gills of the latter maintain bacterial symbionts in a primitive, extracellular location. We studied populations of a species resembling *Thyasira gouldi* in a Northwest Atlantic fjord (Bonne Bay, Newfoundland), and using comparative analyses of 18S and 28S rRNA uncovered a relatively high level of genetic variation with seven defined operational taxonomic units (OTUs). Investigations of gill structure and ultrastructure revealed striking differences among OTUs 1-4, which possess elongated gills that accommodate high numbers of symbiotic bacteria, and OTUs 5-7, whose short gills lack symbionts. The reconstructed 18S+28S phylogeny indicates closer relationships among Bonne Bay *T.* aff. *gouldi* OTUs than between the latter and *T. gouldi* from the eastern Atlantic. *T.* aff. *gouldi* from Bonne Bay form a species complex consisting of at least two sympatric species that possibly diverged post-glacially (<20 thousand years before present) within the Bonne Bay fjord, although this needs further investigation. Such a wide morphological and symbiosis-related disparity, as described here between closely-related but hitherto unrecognized cryptic species suggests that chemosymbiosis itself may influence speciation.

Introduction

Thyasiridae (SF Thyasiroidea, Taylor *et al.*, 2007a) are one of six bivalve families known to have established symbiotic relationships with chemoautotrophic bacteria (Dufour, 2005; Oliver & Taylor, 2012), although not all species within the family are chemosymbiotic (Southward, 1986; Dufour, 2005). Their wide distribution, ranging from shallow coastal sediments to deep sea hydrothermal vents and cold seeps (Payne & Allen, 1991), includes a variety of reducing habitats in which either geologically or biologically produced sulphides can support symbiont metabolism (Fisher, 1990; Dubilier *et al.*, 2008). In all but one chemosymbiotic thyasirid species, the bacterial symbionts are extracellular (Southward, 1986, Fujiwara *et al.*, 2001), residing either in enlarged spaces limited by the microvilli and the cell membrane in the bacteriocyte zone of modified, abfrontally expanded gill filaments, or among the microvilli of abfrontal epithelial cells in gills with shorter filaments (Dufour, 2005). Extracellular chemosymbionts also occur in the gills of mytilids from wood falls (i.e., naturally sunken wood, Duperron *et al.*, 2008); this situation may be an evolutionary precursor to the intracellular symbiosis observed in other bivalves (Dufour, 2005). Ultrastructural evidence indicates that thyasirid gills periodically engulf and digest their symbionts (Dando & Southward, 1986; Southard, 1986; Spiro *et al.*, 1986; Dando & Spiro, 1993), and the nutritional dependence of thyasirids on their bacterial symbionts varies among species. *Thyasira sarsi*, for example, apparently depends extensively on its symbionts, (Dando & Spiro, 1993), whereas *Thyasira (Parathyasira) equalis* likely obtains less nutritional input from symbiont

digestion (Dando & Spiro, 1993; Dufour, 2005); the latter species is considered better adapted to fluctuating environmental conditions (Dufour & Felbeck, 2006), and may be facultatively symbiotic. Thyasirids are ideal study models for the evolution of chemosymbiosis because of the wide variability in symbiont presence and abundance within the family, and because Thyasiridae are thought to represent the most primitive stages of bivalve-bacteria symbiosis (Fisher, 1990; Dufour, 2005).

Species-level identification of thyasirids is challenging because clear diagnostic characters are few and the inferred high intraspecific variation associated with their shell (Taylor *et al.*, 2007a). However, frequent association with organically enriched sites, including gas and oil fields (Oliver & Killeen, 2002), and the occurrence of both symbiotic and asymbiotic species (Dufour, 2005), has renewed interest in the taxonomy and evolution of the Thyasiridae (e.g., Oliver & Sellanes, 2005; Oliver & Levin, 2006; Taylor *et al.,* 2007a; Zelaya, 2009 & 2010; Keuning & Schander, 2010; Keuning *et al.*, 2011). The revised Thyasiridae phylogeny led to its removal from the Lucinoidea and to the designation of Thyasiroidea as a superfamily, because the Thyasiridae formed a highly supported monophyletic clade within the heterodont bivalves, distinct from the Lucinidae and Ungulinidae (Taylor *et al.*, 2007a). Further, the group has been placed at a near basal position within the phylogeny (Taylor *et al.*, 2007a). However, gene sequences have been published in just 20 of 90 described living species (Coan *et al.,* 2000) to date with partial sequences of 18S and 28S nuclear genes from 16 species (Taylor *et al.* 2007a&b; Rodrigues & Duperron, 2011), and partial sequences of the CO1 mitochondrial gene from 4 species (Mikkelsen *et al.,* 2007). Clearly many gaps remain in understanding

the evolutionary history of the family, particularly given that most thyasirid nuclear (18S and 28S) gene sequences represent only one geographic location (and possibly a single individual).

In a preliminary investigation of abundant populations of thyasirids in Bonne Bay, Newfoundland, Canada, two thyasirid taxa were distinguished, based on shell morphology. The first belongs to the subgenus *Parathyasira* (Chapter 1), and the second resembled *Thyasira gouldi* (henceforth referred to as *Thyasira* aff. *gouldi*). Key defining shell characteristics leading to this diagnosis include: 1) an equilateral-ovate outline, 2) a well-defined submarginal and posterior fold, 3) the presence of an auricle, and 4) a narrowly rounded ventral margin, appearing to be slightly angulate in several of the specimens (Oliver & Killeen, 2002). Preliminary analyses of the 18S and 28S nuclear genes of *T.* aff. *gouldi* revealed unexpectedly high variation (~0.42% divergence) in these conserved genes, suggesting that individuals may belong to separate species despite similar morphologies. Species complexes, defined either as groups of closely related species (i.e., cryptic, sibling, or incipient; Knowlton, 1993; Gardner, 1994 & 1996), or as highly variable species based on molecular or other data but are difficult to differentiate using morphological characteristics (for review, see Mikkelsen, 2011), have been discovered in the chemosymbiotic bivalve families Vesicomyidae (Vrijenhoek *et al.*, 1994; Goffredi *et al.*, 2003) and Lucinidae (Taylor & Glover, 2005). However, no species complexes have been described in the Thyasiridae family, probably because of insufficient sampling on a smaller geographic scale.

Here, we describe a *Thyasira* aff. *gouldi* complex comprised of individuals collected from the Bonne Bay subarctic fjord on the west coast of Newfoundland, Canada. This description uses both morphological techniques, including the description of gill filament structure and ultrastructure, and the analysis of partial 18S and 28S nuclear gene sequences. We chose to study those nuclear genes rather than the mitochondrial CO1 gene for two reasons: 1) the majority of thyasirid sequences (~16) available for comparison consist of 18S and 28S fragments; and 2) these genes are useful in phylogenetic studies at various systematic scales because they contain both variable and highly conserved regions (Steiner & Hammer, 2000).

Materials and Methods

Study site

Bonne Bay, a subarctic fjord located in western Newfoundland, Canada ($49^0$30'N $57^0$55'W, **Figure 2-1**: A), is partially separated from the Gulf of St. Lawrence by a 50 m deep sill which retains a deep layer of cold water year round (Conan *et al.*, 1996). The bay is comprised of two "arms", the South Arm (max depth ~100 m) and the East Arm (max depth ~ 230 m) (Conan *et al.*, 1996; Quijón & Snelgrove, 2005), the latter further separated by a shallow (15 m depth) sill (**Figure 2-1**: B, see Chapter 1). Collection of thyasirids was attempted from approximately eight sites within Bonne Bay but found thyasirids at only one of those sites, all located within East Arm: Southeast Arm (S, 49°27'51.46"N, 57°43'09.04"W, 30 m depth), Deer Arm (D, 49°32'43.48"N,

57°50'28.45"W, 30 m depth), and Neddy's Harbour (N, 49°31'21.44"N, 57°52'11.07"W, 15 m depth). Sites D and N are separated by approximately 4 km, sites D and S by 14 km, and sites S and N by 13 km.

Thyasirid collection

The specimens used in this study (N = 198) were collected using a Peterson grab (radius = 10.5 cm, length = 30 cm, volume ~ 2.08×10^4 cm^2) on five separate occasions: October 2009 (O-09), May 2010 (M-10), August 2010 (A-10), April 2011 (Ap-11), and June 2011 (J-11). Thyasirids were separated from sediments using a 1 mm mesh and identified roughly to species based on the key shell characteristics described in Oliver & Killeen (2002). The gills of each individual were carefully dissected out and allocated to either molecular analyses (N = 133) or sectioning (N = 125), and in some cases, to both (N = 60) (as described below). Shell diameter was measured in millimeters for each individual, represented by the length from the anterior to posterior margins.

DNA extraction, PCR amplification and sequencing

We immediately placed gills (N = 133) in 95% ethanol after dissection, and never pooled gills from different individuals together. We isolated and purified DNA from the gill tissue using the QIAgen DNeasy® Blood and Tissue kit, following the spin-column protocol for animal tissues. Polymerase chain reactions (PCRs) used three sets of primers, two for amplifying fragments of the 18S rRNA gene, and one for amplifying a fragment of the 28S rRNA gene: 1) 18S [forward] - GCCAGTAGCATATGCTTGTCTC and 18S

[reverse] AGACTTGCCTCCAATGGATCC (Holland *et al.,* 1991), ~550 bp fragment; 2) 18S-5' [forward] CTGGTTGAT(C/T)CTGCCAGT (Winnepenninckx *et al.,* 1998) and 18S1100R [reverse] CTTCGAACCTCTGACTTTCG (Williams *et al.,* 2003), ~1000 bp; and 3) LSU-5' [forward] TAGGTCGACCCGCTGAAYTTAAGCA (Littlewood *et al.*, 2000) and LSU1600R [reverse] - AGCGCCATCCATTTTCAGG (Williams *et al.*, 2003), ~ 1500 bp. We performed each 25 µL PCR reaction using the Promega PCR Master Mix (Promega Corp.) containing 50 µ/mL Taq DNA polymerase, 400 µM of each dNTPs, 3.0 and 2.5 mM of $MgCl_2$ for 18S and 28S, respectively, and reaction buffer at a pH of 8.5. We used the following thermocycler conditions: initial denaturation at 94 ^{0}C for 4 min, 35 cycles of denaturation at 94 ^{0}C for 30 sec, annealing temperatures of 54 ^{0}C and 52 ^{0}C for 18S and 28S, respectively, and elongation at 72^{0}C for 2 min, with a final elongation at 72 ^{0}C for 5 min. We filtered all amplified products using Acro-Pro® 100K-Omega filters (Pall Life Sciences), and performed sequencing reactions using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems) and electrophoresis on an Applied Biosystems 3730 DNA Analyzer automated capillary sequencer running Sequencing Analysis v. 5.2 Software (Applied Biosystems).

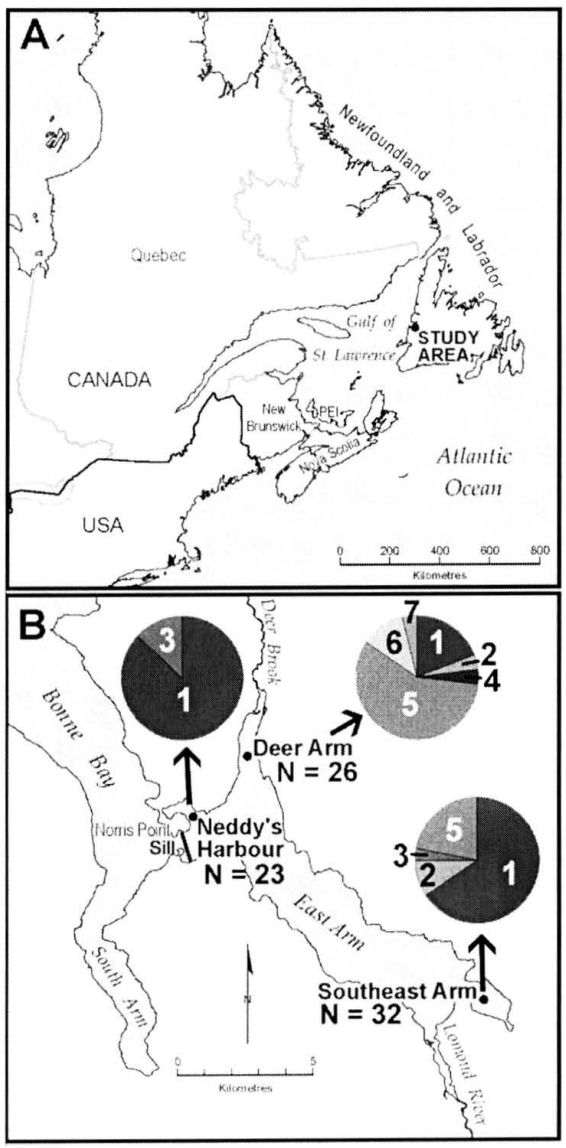

Figure 2-1: Map of study site and surrounding area. A. study area (closed dot) in Newfoundland, Canada. B. Study area (Bonne Bay) with Deer Arm, Neddy's Harbour, and Southeast Arm collection locations. The pie charts at each location show relative proportions of OTUs (1-7) and total number of individuals (N) used in the molecular analysis.

Table 2-1: Species included in phylogenetic analyses. Species collected from Bonne Bay are in bold. Group number for each species given, groups largely based on symbiont presence or absence and gill demibranch number (type). S, symbiotic; A, asymbiotic; P, partially symbiotic; P/A, either partially or asymbiotic; OG, outgroup; 1, one gill demibranch; 2, two demibranchs; and TBD, to be determined.

Species	Group	Desc.	Locality	18SrRNA	28SrRNA
***Thyasira aff. gouldi* OTU 1**	1	S2	Bonne Bay, NL, Canada	TBD	TBD
***Thyasira aff. gouldi* OTU 2**	1				
***Thyasira aff. gouldi* OTU 3**	1				
***Thyasira aff. gouldi* OTU 4**	1				
***Thyasira aff. gouldi* OTU 5**	1				
***Thyasira aff. gouldi* OTU 6**	1	A2			
***Thyasira aff. gouldi* OTU 7**	1				
Thyasira gouldi S.coast UK	2	S2	Mill Bay, UK	JF899224	JF899196
Thyasira gouldi N.coast UK	2		Firth of Forth, UK	AJ581871	AJ581904
Thyasira perplicata	3	S2	Angola	AM392448	AM392432
Thyasira flexuosa	3		Plymouth, UK	AJ581870	AJ581903
Thyasira polygona	3		Northern North Sea	AM774484	AJ581904
Thyasira sarsi	3			AM774485	AM779659
Thyasira methanophila	3		Concepción, Chile	AM392447	AM392431
***Thyasira (Parathyasira)* sp.**	4	A2	Bonne Bay, NL, Canada	TBD	TBD
Thyasira cf. subovata	4		Scotia Ridge, Antarctica	AM392451	AM392435
Parathyasira equalis	4	P2	Gullmarsfjorden, Sweden	AM774482	AM779656
Thyasira sp. VENT	5	S2	Fiji Back Arc Basin (Deep Sea)	AM392452	AM392436
Thyasira sp. REGAB	5		Gulf of Guinea (Deep Sea)	FR716450	FR716451
Mendicula ferruginosa	6	A1	Northern North Sea	AM774483	AM779657
Lepataxinus indusarium	6		Arabian Sea, Pakistan	AM392454	---
Axinulus sp.	6		Scotia Ridge, Antarctica	AM392441	AM392440
Adontorhina cyclia	6		San Diego, California	AM392455	AM392438
Carditamera floridana	7	OG	Manatee Co., Florida, USA	AF229617	---
Eucrassatella cumingii	7		Moreton Bay, Australia	AM774479	AM779653

Phylogenetic analyses

We aligned and compared forward and reverse sequences using Sequencher® (v. 5.0, Gene Codes Corp.) in order to verify bases and then used the online Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990) to match the sequences to closely related sequences in GenBank. We identified operational taxonomic units (OTUs) using MEGA5 (Tamura *et al.*, 2011) by aligning and then grouping individual thyasirid sequences based on polymorphic nucleotide sites; OTUs therefore contained identical sequences (i.e., no base discrepancies). All alignments were executed with default values in MEGA5, using ClustalW (Thompson *et al.*, 1994).

We determined the relatedness between our OTU pairs by calculating the average evolutionary percent divergence (%*d*) for both 18S and 28S fragments and comparing OTUs by constructing a distance matrix using "*p*-distance" as the evolutionary model in MEGA5; thus, the proportion of base discrepancies, represented as a percentage (% divergence), could be compared between each pair of OTUs, accounting for fragment size. We also attempted to determine the relative amount of intraspecific and interspecific variation within the Thyasiridae family by calculating %*d* within and between groups, which consisted of thyasirid 18S and 28S sequences from this study (group 1), the previously published thyasirid 18S and 28S sequences (groups 2-6) and outgroup species (group 7); a list of the species used in each group is given in **Table 2-1**. Groupings were based mostly on shared morphological characteristics (ex. symbiotic vs. asymbiotic, demibranch number) and the previously-published thyasirid phylogeny (Taylor *et al.*, 2007a). We calculated all divergence estimates in MEGA5 using "*p*-distance" as the

method with 2000 bootstrap replicates to generate S.E. values, and treated gaps/missing data with pairwise deletion.

Phylogenetic reconstruction

Using ClustalW in MEGA5, we aligned our OTU sequences, consisting either of 18S only (OTUs 2, 4, 6; ~ 550 bp) or both 18S and 28S fragments (OTUs 1, 3, 5, 7; ~ 2400 bp) with thyasirid 18S and 28S sequences available in GenBank (**Table 2-1).** We identified and removed poorly aligned sites using Gblocks v. 0.91b (Castresana, 2000), basing the phylogenetic reconstruction for OTUs 1, 3, 5 and 7 on a total of 1003 sites for 18S (93% of original alignment) and 1327 sites for 28S (87% of the original alignment). However, only a 550 bp fragment of 18S was available for OTUs 2, 4, 6 because we accidently discarded the extracted DNA from these specimens, and was therefore unavailable for 28S and further 18S amplification with primer set #2.

We constructed two Bayesian trees using MrBayes v 3.1.2 (Huelsenbeck & Ronquist, 2001), one based on the 18S alignment and the other on the concatenated alignment. The General Time Reversible model was used to estimate the 18S only tree (Tavaré, 1986) (GTR). The two models for estimating the concatenated tree were the Kimura 2-parameter (Kimura, 1980) and the GTR for 18S and 28S, based on a model test using the Maximum-Likelihood statistical method in MEGA5 (Nei & Kumar, 2000). The concatenated tree was constructed by partitioning 18S and 28S fragments, allowing us to apply the desired gene-specific models in the analysis. For both trees, we estimated base frequencies and set rate variation to gamma-distribution with four discrete categories and

allowance for invariant sites. We ran data sets twice, with 500,000 (18S only) or 1,000,000 (18S + 28S) generations each and a sampling frequency of once every 100 generations (both trees), basing the consensus tree on the combination of the remaining trees from each run (trees in total: 7500, 18S; 15000, 18S+28S). Support values associated with the nodes of both trees were based on posterior probabilities (PP) calculated in MrBayes.

Scanning Electron Microscopy of Shells

To obtain images of the shells and to measure prodissoconch size, we carefully cleaned shell valves (N = 10) in water and mounted them on stubs for observation with an FEI Quanta 400 scanning electron microscope under variable pressure mode. We then measured prodissoconch diameter using the software ImageJ (Abràmoff *et al.*, 2004).

Light and Transmission Electron Microscopy of Gills

We retained one gill for morphological analysis (N = 125). Gills were fixed in 2.5 % gluteraldehyde in 0.1 M sodium cacodylate buffer for 24 hours before transfer to a 0.1 M sodium cacodylate buffer. We subsequently stained the gills with 1% osmium tetroxide in the same buffer, and dehydrated in an ascending ethanol series before embedding them in EPON resin. We made semi-thin (1 µm) sections using a LKG Bromma 8800 ultramicrotome, and stained them with 1% toluidine blue in 1% sodium borate for light microscopy. We also made ultra-thin sections (60 nm) which we post-stained using

uranyl acetate and lead citrate, and observed using a Philips 300 transmission electron microscope.

Statistical analyses

To determine whether evolutionary percent divergence (%*d*) was significantly different between symbiotic (1-4) and asymbiotic (5-7) OTUs, we conducted a standard least squares (LS) analysis using the statistical program JMP (v. 10, SAS Institute Inc., Cary, NC, USA) where %*d*, calculated in MEGA5 as previously described, was treated as the response variable, and the type of group (i.e., within-group: symbiotic or asymbiotic, between group: symbiotic versus asymbiotic) comparisons was treated as the explanatory variable. All assumptions were met on the residuals of this analysis. Once the analysis was discovered to be significant, we used a Tukey's HSD *post-hoc* test to specifically determine which groups were significantly different.

Similarly, we conducted another LS analysis based on the sequences obtained in this study and other thyasirid sequences, as listed in **Table 2-1**) to determine whether group comparisons (explanatory variable, 29 categories in total) were significantly different, including both within- and between-group comparisons (i.e., X*X and X*Y). In order to meet assumptions on the residuals, the response variable %*d* was square-root transformed. Again, we used a Tukey's HSD test to determine what groups in particular were significantly different.

To determine whether shell diameter was significantly different between symbiotic versus asymbiotic individuals, we conducted a third LS analysis in which shell

diameter was treated as the response variable while symbiotic condition (two categories: sym. vs. asym.) was treated as the categorical explanatory variable. Assumptions were met on the residuals, and a *post-hoc* test was not conducted (i.e., there were only two groups to compare).

Results

Molecular descriptions: OTUs

Out of the 133 individuals used in the molecular analysis, 81 18S fragments (range: 553 bp for OTUs 2, 4, & 6 to 1001 bp for OTUs 1, 3, 5, & 7) were successfully sequenced, all most closely matching *Thyasira gouldi* (GenBank accession number JF899224), with a sequence similarity of 99%. Out of the individuals in which 18S fragments were obtained, 59 28S fragments (range: 1151 bp for OTU 7, 1428 for OTUs 1, 3, & 5) were successfully sequenced, all most closely matching *Thyasira gouldi* (JF899196) with a slightly lower sequence similarity (98%). In total, we defined seven distinct OTUs (1-7) in which 4 sites (out of 1001) and 13 sites (out of 1428) were polymorphic within the 18S and 28S fragments, respectively (**Table 2-2**). The same individuals grouping together for the 18S fragment also grouped together for the 28S fragment, with exception of individuals associated with OTUs 5 and 7, differing in the 18S fragments by a single base pair (position 233), whereas no base differences were evident in the 28S fragment. Importantly, we lacked 28S sequences corresponding to 18S OTUs 2, 4, 6 (as indicated by [?] in **Table 2-2**), and comparisons for these OTUs were

based only on a ~550 bp region of the 18S fragment (18S primer set #1). Unfortunately, OTUs 2, 4, 6 were only found during a single collection period (O-09), and mostly at a single site (D), despite multiple sampling occasions, so we could not obtain additional material for 28S amplification.

Phylogenetic reconstruction

For both trees (**Figure 2-2** & **Figure 2-3**), all the *Thyasira* aff. *gouldi* OTUs of the present study strongly grouped together (97-99% support value) within the *T. flexuosa*, *T. polygona*, and *T. gouldi* clade, and appeared to be more closely related to *Thyasira gouldi* from Mill Bay, on the south coast of the United Kingdom, in the concatenated tree (**Figure 2-3**). The concatenated tree highly supported the *T.* aff. *gouldi* clade (97% PP), in contrast to moderate support in the 18S only tree (57% PP). Both trees strongly grouped together OTUs 5-7 (91-95% PP), whereas OTUs 1-4 were less resolved and formed a polytomy. OTUs 2 and 4 strongly grouped together (83% PP) within the concatenated tree, but grouped moderately (58% PP) together in the 18S only tree. In terms of phylogenetic positioning, OTUs 1-4 occupied a more basal position than OTUs 5-7, in both trees. It is noteworthy that OTUs 2, 4 and 6 consisted only of a 550 bp region of the less variable 18S fragment, with no 28S sequence data available for these specimens; their position in the phylogeny would be less reliable.

Table 2-2: OTU descriptions showing total number of individuals (N), sites collected (Deer Arm, D; Neddy's Harbour, N; Southeast Arm, S) with number of individuals collected from each site, polymorphic nucleotide sites with position given, and distance matrix comparing the average evolutionary percent divergence (%*d*) between each OTU. Dots in 18S/28S columns indicate same base as top row, (?) indicates missing data. Distance matrix based on concatenated 18S and 28S OTU sequences, in which *d* is represented as a percent; %*d* was calculated using the 'p-distance' model in MEGA5, treating gaps/missing data with pairwise deletion. S.E. estimates on each comparison are given above the diagonal in italics. Shading indicates lowest (red) to highest (green) levels of %*d*.

OTU	N	Collection site			18S										28S							OTU	Distance matrix						
		D	N	S	229	233	283	544	729	746	773	781	788	790	797	824	885	971	990	995	1026		1	2	3	4	5	6	7
1	46	5	20	21	C	C	T	T	C	C	T	G	C	T	A	C	T	C	C	T	C	**1**	---	*0.18*	*0.14*	*0.30*	*0.15*	*0.39*	*0.15*
2	4	1	0	3	.	.	.	G	?	?	?	?	?	?	?	?	?	?	?	?	?	**2**	0.18	---	*0.30*	*0.19*	*0.39*	*0.32*	*0.32*
3	4	0	3	1	.	T	.	.	T	.	.	A	T	C	G	.	A	A	T	C	T	**3**	0.45	0.36	---	*0.18*	*0.11*	*0.32*	*0.12*
4	1	1	0	0	.	T	.	G	?	?	?	?	?	?	?	?	?	?	?	?	?	**4**	0.36	0.18	0.18	---	*0.32*	*0.26*	*0.40*
5	21	15	0	6	T	T	A	.	T	T	C	A	.	.	.	A	A	A	T	C	T	**5**	0.54	0.72	0.33	0.54	---	*0.18*	*0.04*
6	3	3	0	0	T	T	A	G	?	?	?	?	?	?	?	?	?	?	?	?	?	**6**	0.72	0.54	0.54	0.36	0.18	---	*0.31*
7	2	1	0	1	T	.	A	.	T	T	C	A	.	.	.	A	A	A	T	C	T	**7**	0.51	0.56	0.38	0.74	0.04	0.37	---

Evolutionary divergence

The least squares (LS) analysis on evolutionary percent divergence (%*d*) between the OTUs of this study, as estimated in MEGA5, showed an overall significant difference when comparing *T. aff gouldi* symbiotic with asymbiotic OTUs [$F(2, 18) = 11.40$, $p = 0.0006$]. The Tukey's HSD *post-hoc* test revealed that %*d* between symbiotic (1-4) and asymbiotic (5-7) OTUs is significantly higher (0.54 %*d* average) compared to the within-group %*d* among symbiotic OTUs (0.29%*d*) or asymbiotic OTUs (0.20%*d*), the latter two which were not significantly different (**Table 2-2**).

The overall result of the LS analysis on sequences from the Thyasiridae family and outgroup species (see **Table 2-1** for group descriptions) showed that significant differences within and between groups were present [$F(28, 271) = 46.0$, $p < .0001$]. The Tukey's HSD *post-hoc* test revealed that within-group percent divergence (%*d*) for all *T.* aff. *gouldi* individuals from Bonne Bay (group 1) was significantly lower (0.42% average) than the symbiotic thyasirids with 2 demibranchs (group 3, 2.50%*d*), the asymbiotic or partially symbiotic thyasirids with 2 demibranchs, (group 4, 3.42%*d*), and the symbiotic thyasirids from the deep-sea (group 5, 20.59%*d*); however, the within-group %*d* of the Bonne Bay thyasirids was not significantly different from the previously published *T. gouldi* sequences (group 2, 1.18%*d*), the asymbiotic thyasirids with one demibranch (group 6, 1.41%*d*), and the outgroup sequences (group 7, 2.72%*d*) (**Table 2-3**).

The *post-hoc* test also revealed that our OTUs are least divergent (i.e., not significantly different) compared to the a) previously published *T. gouldi* sequences

(group 2, 1.12%*d*), b) symbiotic thyasirids with two demibranches (group 3, 2.49%*d*), and c) asymbiotic thyasirids with one demibranch (group 6, 2.49%*d*) (**Table 2-3**). The two deep-sea thyasirids (group 5) were the most divergent across all between-group comparisons (16.56 %*d* average), significantly outweighing the outgroup species (group 7, average between-group %*d* = 8.47) (**Table 2-3**).

Shell characters and size

Of the 198 specimens examined in this study, shell outline (**Figure 2-4**: A, B) was equilateral-ovate, slightly higher than long, and bisinuate. A weakly projecting auricle defined the posterior region, with a submarginal sulcus forming a marginal sinus and a posterior fold forming a posterior sinus. The hinge plate lacked a clear cardinal tooth (**Figure 2-4**: B). The large prodissoconch averaged of 196 µm in diameter (size range: 180-222 µm, N = 10, **Figure 2-4**: C). The size range of all *Thyasira* aff. *gouldi* individuals was of 1.0 - 5.2 mm. The standard least squares analysis revealed that the shell diameter of symbiotic individuals (OTUs 1-4) was significantly greater than asymbiotic individuals (OTUs 5-7) (symbiotic: 3.14 ± 0.11 mm; asymbiotic: 2.60 ± 0.16 mm) [$F(1, 58) = 7.72$, $p = 0.0074$].

Table 2-3: Estimates of average evolutionary percent divergence (%*d*) for sequence pairs within groups (second column) and between groups (distance matrix). Bold numbers indicate group, italicized numbers above diagonal in distance matrix are S.E. values for each pair. All calculations conducted in MEGA5, using the "p-distance" model in order to account for variable fragment sizes. Refer to **Table 2-1** for list of species involved in each group. Values surrounded by a thick box are significantly different ($p < 0.001$) from our *T. aff. gouldi* thyasirids, based on the Tukey's HSD *post-hoc* test.

				Distance Matrix						
Group/description	%*d*	S.E.	OTU	**1**	**2**	**6**	**7**	**3**	**4**	**5**
1. T. aff. gouldi (BB)	0.42	0.18	**1**	---	*0.26*	*0.26*	*0.58*	*0.27*	*0.29*	*0.70*
2. T. gouldi (UK)	1.18	0.24	**2**	1.21	---	*0.29*	*0.52*	*0.20*	*0.28*	*0.63*
6. Thyasiridae (1A)	1.41	0.21	**6**	2.49	2.78	---	*0.50*	*0.22*	*0.21*	*0.59*
7. Outgroup	2.72	0.52	**7**	6.38	6.59	6.89	---	*0.47*	*0.47*	*0.71*
3. Thyasira sp. (S2)	2.50	0.21	**3**	2.49	2.32	2.72	6.91	---	*0.21*	*0.62*
4. Thyasira sp. (P/A2)	3.42	0.3	**4**	3.11	3.23	2.69	7.20	3.11	---	*0.66*
5. Thyasira. sp. (S2, DS)	20.59	0.82	**5**	15.56	15.69	16.14	16.88	16.93	18.18	---

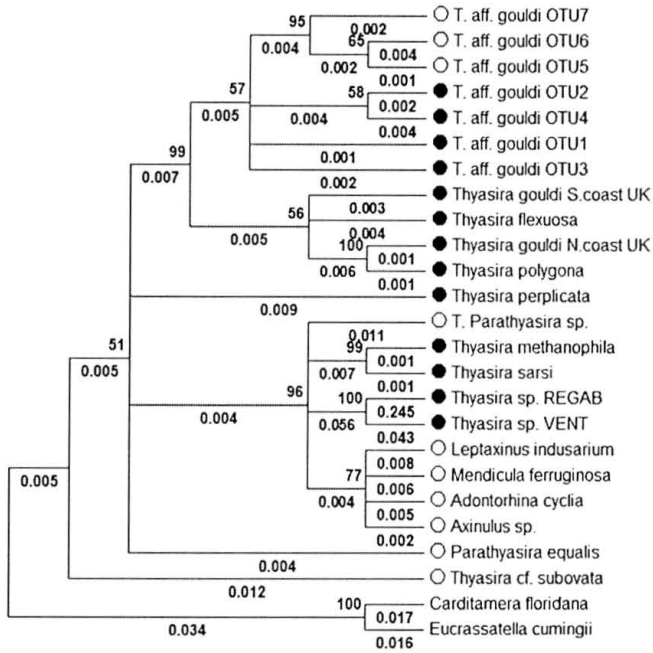

Figure 2-2: 18S rRNA tree showing phylogeny of thyasirids collected in this study (*Thyasira* aff. *gouldi* OTUs1-7) and other species from various localities (Table 2-1). Outgroup composed of species *Carditamera floridana* and *Eucrassatella cumingii*. Support values (above branch) calculated as posterior probabilities shown next to branches, unless < 50% in which branches are collapsed. Branch lengths (below branch) in units of # base substitutions per site. Closed circles indicating symbiotic species, open circles indicating partially symbiotic, asymbiotic, or undetermined species.

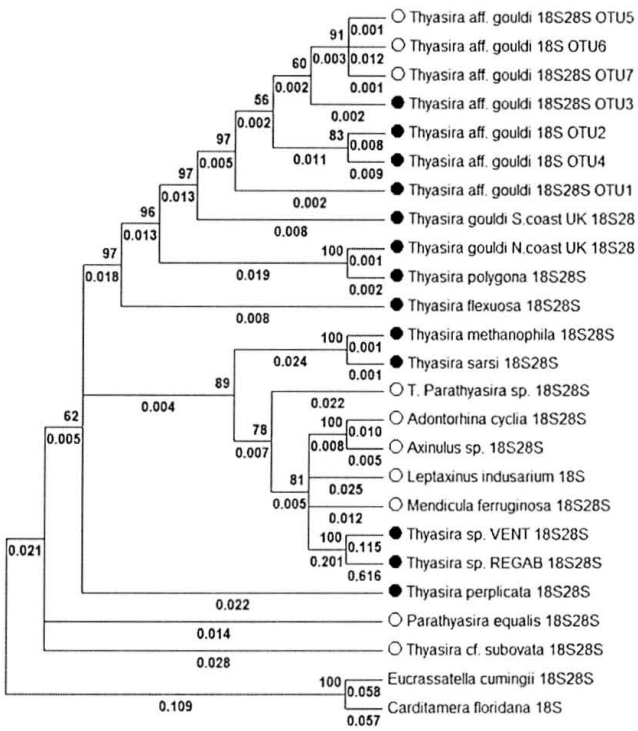

Figure 2-3: Concatenated 18S + 28S Bayesian tree showing phylogeny of thyasirids collected in this study (*Thyasira* aff. *gouldi*) and other species from various localities (all accession numbers in (Table 2-1). Both 18S and 28S sequences were available for all species, unless indicated (18S only). *Carditamera floridana* and *Eucrassatella cumingii* comprised the outgroup. Support values (above branch) calculated as posterior probabilities shown next to branches, unless < 70% in which branches are collapsed. Branch lengths (below branch) in units of # base substitutions per site. Closed circles indicating symbiotic species, open circles indicating either partially symbiotic or asymbiotic species.

Gill morphology

Out of the total specimens examined (N = 198), 60 had corresponding molecular (all seven OTUs) and morphological data available. From this subset of individuals, we observed two different gill filament morphologies (**Figure 2-5**: A, B). All individuals from OTUs 1-4 (N = 40) had opaque, pink to white gills, with 'type 3' morphology filaments as originally described by Dufour (2005) (**Figure 2-5**: A, **Table 2-4**). These gills are abfrontally expanded, with a clear bacteriocyte zone abfrontal to the frontal ciliated zone, and TEM observations revealed large numbers of extracellular symbionts (**Figure 2-5**: C, **Table 2-4**). In contrast, the shorter, translucent, 'type 2' gills, from all individuals with OTUs 5-7 (N = 20) had conspicuously lacked a bacteriocyte zone (**Figure 2-5**: B, **Table 2-4**); fewer cells characterized filaments in these individuals in the region abfrontal to the frontal ciliated zone. The abfrontal epithelium was pseudostratified, with apical cells overlying more basal cells, the latter containing numerous enlarged mitochondria (**Figure 2-5**: D). Symbiotic bacteria were absent from OTUs 5-7 and we observed very little staining with toluidine blue in their abfrontal cells.

Internal anatomy

The specimens of this study (N = 198) had gills with two demibranchs, varying in colour and relative thickness among individuals, a mantle margin thickened at the anterior end, an elongated, vermiform foot, and a single mass of digestive diverticula (i.e., not as branched as in other thyasirid species) (**Figure 2-4**: D).

Figure 2-4: Morphology of *Thyasira* aff. *gouldi* collected from Bonne Bay, Newfoundland. A. Outer view of the left shell valve, with a weakly projecting auricle (a), a well-defined submarginal sulcus (ss) forming a marginal sinus, a distinct, yet rounded, posterior fold (pf), and a rounded ventral margin (vm). B. Inner view of the right valve, revealing the absence of dentition along the hinge plate (hp). C. Scanning electron micrograph of the larval shell (~ 191 µm diameter). D. Internal anatomy, showing a gill (g) with two demibranchs, the digestive diverticula (d), foot (f), anterior adductor (aa), posterior adductor (pa) and mantle margin, thickened at the anterior end (m).

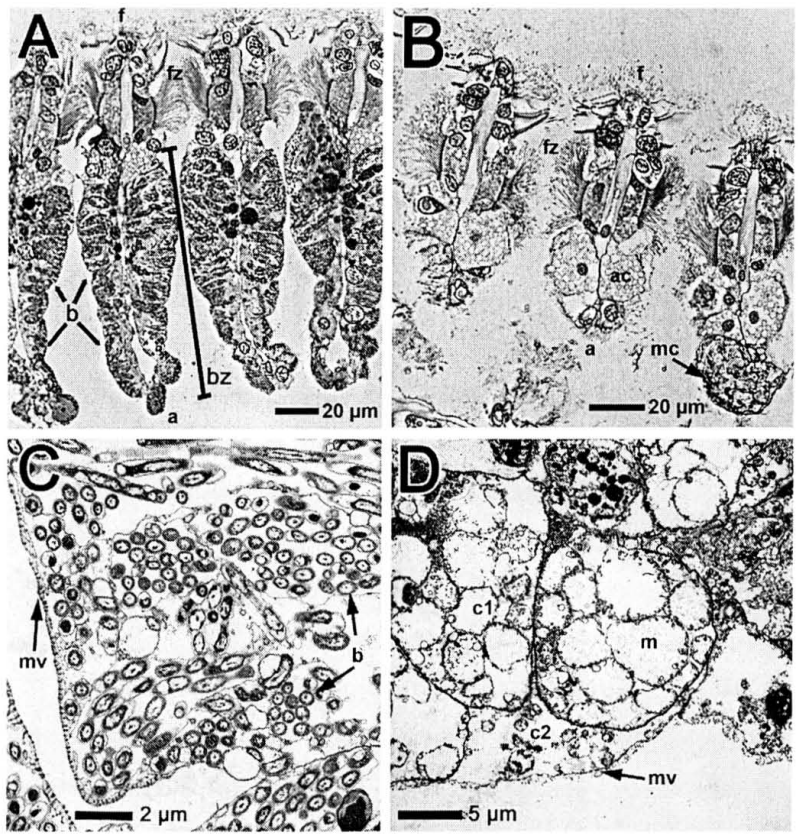

Figure 2-5: Light and electron micrographs of 'type 2' (B & D) and 'type 3' (A & C) gill filaments. A. *Thyasira* aff. *gouldi* symbiotic OTU (1-4). Light micrograph of a semi-thin, transverse section of four gill filaments, shows frontal zone (fz), bacteriocyte zone (bz), and bacteria (b), seen as darker stained areas within bacteriocytes. a: abfrontal end of a filament; f: frontal end of a filament. B. *Thyasira* aff. *gouldi* asymbiotic OTU (5-7). Light micrograph of a semi-thin, transverse section of three gill filaments. Note absence of abfrontal expansion and bacteriocytes. a: abfrontal end of filament; ac: abfrontal cell; f: frontal end of filament; fz: frontal ciliated zone; mc: mucocyte C. *Thyasira* aff. *gouldi* symbiotic OTU (1-4). TEM of cells in the abfrontal zone of a gill filament, show an abundance of bacteria (b), which are maintained extracellularly, in pockets limited by extensions of host cell cytoplasm bearing microvilli (mv). D. *Thyasira* aff. *gouldi* asymbiotic OTU (5-7). TEM of cells in the abfrontal zone of a gill filament, show an absence of bacteria. The epithelium is pseudostratified, with more apical cells (c2) overlying basal cells (c1) containing large mitochondria (m). mv: microvilli.

Table 2-4: Corresponding molecular (OTUs) and gill filament morphology, indicating number of individuals (N), mean shell size and standard error (S.E.) based on a standard least squares analysis, gill type (sensu Dufour, 2005), and presence (+) or absence (-) of symbionts.

OTU	N	Shell Size (mm)	95% CI		Gill Type	Symbiont
		LS mean	Lower	Upper		
1	32	3.01	2.81	3.21	3	+
2	4	4.25	3.70	4.80	3	+
3	3	2.43	1.78	3.08	3	+
4	1	5.00	3.88	6.12	3	+
Ave. (1-4) N tot = 40		**3.14**	**2.92**	**3.36**		
5	16	2.47	2.20	2.74	2	-
6	2	3.50	2.72	4.28	2	-
7	2	2.75	1.97	3.53	2	-
Ave. (5-7) N tot = 20		**2.60**	**2.29**	**2.91**		

Geographic distribution

The distribution of OTUs within Bonne Bay, as depicted in **Figure 2-1**: B, showed that Deer Arm (D) was dominated by individuals with the asymbiotic morphotype (OTUs 5-7), whereas symbiotic morphotypes dominated Southeast Arm (S, OTUs 1-4) and were the only morphotype collected from Neddy's Harbour (N, OTUs 1&3). Thus, asymbiotic morphotypes were limited to two of the three sites, whereas symbiotic morphotypes were more widespread (**Figure 2-1: B**).

Discussion

General affiliation: Thyasira aff. *gouldi from Bonne Bay, NL, Canada*

For several reasons, we initially thought that all specimens collected and described in this study belonged to a single species, most closely resembling *Thyasira gouldi* (Philippi, 1845). First, the Bonne Bay thyasirids share key shell characteristics with *T. gouldi* described from the eastern and western Atlantic (Gould & Binney, 1870; Oliver & Killeen, 2002), most notably the presence of a large prodissoconch, previously defined as a key distinguishing character for this species (Oliver & Killeen, 2002). The latter measures between 205-270 μm in *T. gouldi* individuals (N = 55) from Norway, Faeroe Islands, New England and Greenland (Oliver & Killeen, 2002), but is slightly smaller in Bonne Bay specimens. Second, the internal anatomy of Bonne Bay *T.* aff. *gouldi* resembles described *Thyasira gouldi* specimens from the eastern Atlantic (Oliver & Killeen, 2002) and we are unaware of descriptions of the internal anatomy for western

Atlantic specimens. Both the Bonne Bay and eastern Atlantic specimens have relatively enlarged gills with two demibranchs, a long, vermiform foot, and unbranched digestive diverticula (slightly smaller in the Bonne Bay specimens). Also, *Thyasira gouldi* maintains chemosynthetic bacterial symbionts within the bacteriocytes of its abfrontally expanded 'type 3' gills (Dufour, 2005). Initial transmission electron micrographs of gills from a subsample of Bonne Bay specimens revealed similar 'type 3' gills with abundant bacterial symbionts. Finally, the 18S and 28S sequences of the Bonne Bay species most closely resemble (> 98% similarity) those of *T. gouldi* from the south coast of the UK.

Furthermore, both the geographical and depth distribution of *Thyasira gouldi* corresponded to our sampling locations. *T. gouldi* was initially collected in deep water off Massachusetts (Gould, 1841; Gould & Binney 1870), and has since been reported as panarctic in distribution at < 50 m depth (Ocklemann, 1958) based on populations sampled from Scottish Sea Lochs, Southern Norwegian fjords, the Firth of Forth, and the Southwest coast of the United Kingdom (Oliver & Killeen, 2002; Distel *et al.,* 2011). Thus, the presence of *T. gouldi* in 15 - 30 m depth in the Bonne Bay subarctic fjord would not be surprising. The three collection sites from Bonne Bay (D, N, S) contained glacial sediments exhibiting organic enrichment (for OM% data at each site, see Chapter 1); terrestrial organic matter carried by river run-off settles into confined channels of the bay, particularly at sites D and S where Deer Brook and the Lomond River flow into the northern and southern parts of these arms, respectively. *T. gouldi* usually occurs in organically enriched clay-grade sediments (Oliver & Killeen, 2002, Wlodarska-Kowalczuk, 2007).

Molecular and morphological variation in T. aff. gouldi individuals

When we integrated molecular data and associated gill filament morphology, two distinct groups within the *T. aff. gouldi* group appeared: OTUs 1-4 possessed 'type 3' gills with abundant bacteria, whereas OTUs 5-7 possessed a completely different type of gill morphology – 'type 2' gills without symbionts. Correspondingly, both of the phylogenies constructed placed the asymbiotic OTUs in a strongly supported group, separating them from the symbiotic OTUs. Further, when comparing between OTUs 1-4 and 5-7, evolutionary %*d* was around twice as high (0.54%) compared to the %*d* within OTUs 1-4 (0.29%) and OTUs 5-7 (0.20%). Based on these results, we argue at least two putative species are present for several reasons. First, the level of variation in both 18S + 28S fragments (4 variable bases in 18S, 13 in 28S, **Table 2-2**) is deemed too high to represent divergence within a bivalve species (Distel, 2000; Lorion, *et al.*, 2009, Brissac *et al.*, 2010), especially considering that 18S and 28S are highly conserved genes (Hillis & Dixon, 1991). For example, a previous study (Brissac *et al.*, 2010) reported levels of divergence within an undescribed *Thyasira* sp. that were over four-times lower (0.1% in 28S, no differences in 18S) compared to the levels reported here (~0.4%). Second, consistency in OTU groupings across multiple genes (i.e., 18S and 28S) increased the likelihood that OTUs represent distinct species (Avise & Ball, 1990). Third, we often collected different OTUs from the same grab sample; thus, the high level of genetic variation between sympatric OTUs suggests reproductively isolated species with no hybridization, although this needs to be investigated further using species-diagnostic co-dominant markers (Innes, D., & Marshall, D., pers. comm).

In attempt to determine the relative levels of interspecific versus intraspecific variation in the thyasirid family, we compared the 18S and 28S sequences of operational taxonomic units (OTUs) from this study to those of other thyasirids and found little evidence of sufficient divergence to separate species (as opposed to subspecies). For example, the level of %*d* within our OTUs was similar (i.e., not significantly different) to the previously published *T. gouldi* sequences, initially suggesting our OTUs represent subspecies. However, the within-group %*d* containing species of separate genera (group 6) was also similar to the level we found for our OTUs, suggesting a greater level of divergence is represented within *T.* aff. *gouldi*. Also, all comparisons regarding the symbiotic thyasirids from the deep-sea (group 5) suggest that these sequences are either incorrect (i.e., typos), or are misaligned (i.e., contain sequences from other parts of the fragments not present in the alignment), making it more difficult to define an appropriate level of divergence within the Thyasiridae family.

Consideration of the divergence of thyasirids suggests either that the level of variation of 18S and 28S genes may be insufficient to distinguish among species of this group (Lorion *et al.*, 2009; Brissac *et al.*, 2010) or that some published sequences may misidentify the associated species. Thyasirids are often misidentified because their identification relies on weakly delineated shell characters, and shell morphology is often confounded by convergent or parallel evolution (Steiner & Hammer, 2000). Therefore, thyasirid identifications should utilize both molecular data and a wide range of morphological characteristics (Taylor *et al.,* 2007a), and importantly, comparing these analyses to the voucher specimens previously sequenced and described (Mikkelsen *et al.*,

2007). It is important to mention also that many comparisons in the analysis involved incomplete sequence fragments, which would act to decrease the amount of data used in the comparison. For example, if a 1400 bp fragment is compared to a 550 bp fragment, MEGA5 would only compare the overlapping 550 bp because missing data has been treated with "partial deletion". Therefore, interpretations on within and between-group comparisons may be confounded by lack of information for some sequences (OTUs 2,4 and 6), while others have full coverage (OTUs 1,3,5,and 7).

In any case, constraining the acceptable variability within a single thyasirid species and determining the exact number of cryptic *T.* aff. *gouldi* species in Bonne Bay goes beyond the scope of this study, especially because several of the OTUs exhibited low sample sizes (**Table 2-2**). The investigation of mitochondrial genes (i.e., 16S, CO1) might clarify the relatedness among *T.* aff. *gouldi* OTUs, being used in previous studies to identify sister species (see Mikkelsen *et al.*, 2007). Unfortunately, despite the numerous attempts made to obtain CO1 sequences, amplifications were not successful. In addition to CO1, the internal transcribed spacer region of ribosomal genes (Hillis & Dixon, 1991) may shed more light on whether hybridization can occur among the OTUs of this study. However, given the strong morphological (type 2 vs. 3 gills) and corresponding molecular evidence (asymbiotic OTUs forming their own highly supported group), we believe *T.* aff. *gouldi* individuals from Bonne Bay form a cryptic species complex and propose possible mechanisms of speciation in this group in the following sections.

Phylogenetic interpretations of species complexes

Species complexes are more common than previously thought, most likely because advances in molecular methods "correct" for taxonomic ambiguity, and the recent molluscan literature revealed or resolved several species complexes using molecular data (Mikkelsen, 2011). Patterns in the phylogenetic reconstruction of closely related species may reveal radiation events where innovations driving the radiation can be inferred. For example, Mikkelsen (2011) superimposed the numbers of recognized species per Recent bivalve superfamily and identified disparately sized sister pairs (e.g., Lucinoidea with 500 species versus Galeommatidae + Pholadoidea with 30 and 170 species, respectively). Specialized adaptations associated with the more speciose sister taxa (in Luciniodea: harboring sulphide-oxidizing bacteria in gills, allowing the colonization of reducing environments), could have driven the observed radiation.

The phylogenetic analysis of closely related species can also reveal strongly supported polytomies of several species, often indicating either an "unacknowledged species complex" or "rapid ongoing speciation" (Mikkelsen, 2011). For example, Mikkelson (2011) interprets the highly supported polytomy within the Lucinidae family, "Lucinid clade B + Phacoides" (Williams *et al.,* 2004), as rapid speciation likely driven by the specialized location in which lucinids harbour their symbiotic bacteria. The possibility that the observed polytomies in our analysis result from a lack of sequence information to distinguish between OTUs cannot be ruled out; 3 of the 7 OTUs had only a 550 bp 18S fragment available, which would greatly reduce the amount of informative sites used pairwise comparisons.

Due to the strong clustering and the more derived position of asymbiotic compared to symbiotic OTUs in our analysis, however, we felt it important to discuss the possibility that symbionts themselves may be driving speciation, although further evidence is needed to support this statement.

Potential mechanisms driving speciation

Symbiotic thyasirids are not obligately dependent on symbionts for nutrition because they also suspension feed (Dufour & Felbeck, 2006); thyasirids maintained in laboratory experiments lose their symbionts as a response to changes in either sulphide concentrations or rate of symbiont digestion by the clam (Dufour & Felbeck, 2006). Symbiont abundance also varies *in situ*: *T.* aff. *gouldi* specimens from the Bonne Bay fjord showed a seasonal, cyclical trend in symbiont abundance, most likely associated with temporal differences in symbiont digestion rate, reliance on particulate feeding, and sediment sulphide levels (J. Laurich & S. Dufour, in prep.). Sulphide availability, therefore, directly affects symbiont production, and indirectly, the host (Goffredi & Barry, 2002). Therefore, the idea that a subpopulation of asymbiotic thyasirids may arise from an initially symbiotic population is not completely far-fetched. In other words, if conditions are persistently unfavorable for symbionts (i.e., low sulphide), their abundance may decrease without necessarily decreasing that of their hosts'. Further, the behavioral, physiological, and morphological consequences expected to accompany symbiont loss may act to decrease gene flow between symbiotic and asymbiotic thyasirids. For example, asymbiotic OTUs were never found in Neddy's Harbour (N, one of the three sites

thyasirids were commonly found in BB, **Figure 2-1**: B). At the shallower N site (~15m depth), temperature and salinity vary greatly (Chapter 1), and OM% and sulphide concentrations vary strongly in sediments composed mostly of rocks and sand (Chapter 1). Anthropogenic activities, including dredging, also affect this site more than sites S and D (R. Hooper, pers. comm.). Similarly, individuals identified as *Thyasira (Parathyasira)* sp. (Chapter 1, another asymbiotic species with 'type 2' gills, were absent at site N, while they were present at the other two sites. Thus, conditions at N might limit the ability of asymbiotic individuals to successfully colonize this area, subsequently limiting gene flow between populations.

Additionally, the degree of environmental patchiness of ecologically relevant parameters (sulphide and organic matter) may be important for promoting speciation in the *T.* aff. *gouldi* complex. Given the fine-scale temporal and spatial patchiness at the three study sites (D, N, S), where localized "patches" of sulphide were interspersed with zones of low to undetectable sulphide concentrations (pers. observation, see Chapter 1 for average sulphide levels), some individuals of the ancestral *T.* aff. *gouldi* species likely colonized high sulphide areas while others ended up in low sulphide zones within Bonne Bay. Patchiness may facilitate speciation associated with symbiont loss in thyasirids: in sites where both symbiotic and asymbiotic OTUs co-occurred (i.e., S and D), localized areas of sulphide might be analogous to "islands" or microniches, that may act to greatly reduce gene flow across areas of unsuitable habitat. Additionally, we predict further reduction in gene flow between patches (even on a decimeter scale) if the reproductive strategy of *T.* aff. *gouldi* from Bonne Bay mirror those in *T. gouldi* from a Scottish sea

loch, which are known for direct development and low dispersal (Blacknell & Ansell, 1974).

However, evidence for reduced gene flow between OTUs is lacking in this analysis; the co-occurrence of symbiotic and asymbiotic OTUs may simply be due to secondary contact between two closely-related species, and not necessarily speciation driven by symbiosis or environmental patchiness. In a previous study, two closely-related chemosymbiotic clam species (*Calytogena kilmeri* & *C. pacifica*) occurred in sympatry because they could exploit microniches due to difference in their ability to uptake sulphide; higher affinity of sulphide uptake in *C. pacifica* allows them to thrive within areas of lower sulphide, whereas lower affinity in *C. kilmeri* limits their distribution to areas of higher sulphide levels (Goffredi & Barry, 2002). Thus, the observed patchiness, or microniches, were not responsible for promoting speciation, but for allowing two ecologically-similar species to co-exist.

The driving force of speciation in the *T.* aff. *gouldi* complex may be better understood based on whether divergence originated in Bonne Bay, or if it occurred elsewhere. In regards to the former, a greater case for speciation driven by symbiosis could be made; the observed patchiness in sulphide directly determines the abundance symbionts, which indirectly impacts the host and may have evolutionary consequences. Therefore, the ecological consequences of having symbionts (or not having symbionts) could act to set up barriers of gene flow, promoting speciation within a relatively close spatial range. However, if divergence occurred outside the bay, then the colonization of Bonne Bay by different OTUs would be facilitated by patchiness at the same site, and in

some cases, within the same grab, because OTUs are not competing for the same resources. Additional sampling across a greater geographical range would aid in determining if OTUs diverged within or outside of Bonne Bay.

Conclusion

Using a combination of molecular analyses and morphological characterization, we demonstrate the presence of a cryptic species complex consisting of at least two thyasirid species (one symbiotic, the other asymbiotic). It would be important to further investigate speciation in the group, specifically whether divergence originated in Bonne Bay, and if it is driven by symbiont loss in the asymbiotic group (OTUs 5-7). The number of species involved in this complex remains unresolved, and the study of additional sequences, including mitochondrial genes, might be useful for resolving the phylogeny of such closely related species (Hillis & Dixon, 1991). Cryptic species can easily be overlooked because morphological traits such as shell form are often fairly variable within a thyasirid species (Oliver & Killeen, 2002). In addition, the presumed wide (i.e., amphi-Atlantic) distribution of many thyasirids should be re-assessed in light of the differences observed here in gene sequences from apparent conspecifics spanning the Atlantic Ocean. The striking differences we show in gill filament morphology underscore the relevance of this organ as a species diagnostic character for certain groups. Symbiosis can both directly affect gill morphology, with profound ecological and, possibly, evolutionary consequences for the host.

Chapter 3: Lack of host-specificity and co-speciation between closely related thyasirids (Bivalvia: Thyasiroidea) and their extracellular bacterial endosymbionts

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Abstract

The Thyasiridae (Bivalvia: Thyasiroidea) is one of the few chemosymbiotic bivalve families that includes both symbiotic and asymbiotic species. Symbiosis appears to be less well developed in thyasirids than in other bivalve groups; thyasirid symbionts are extracellular, vary among species in their abundance and nutritional importance, and are likely acquired from the environment at each generation. For these reasons, assessing the extent of host-specificity and co-speciation in thyasirids and the nutritional importance of symbionts, could provide valuable information on early stages of symbiosis evolution. We demonstrate that co-occurring sibling thyasirid species can share the same bacterial phylotype, and individuals within a single host species associate with different bacterial phylotypes, indicating low host-specificity by the bacteria. Further, independence between host and symbiont phylogenies does not support co-evolution. We propose a lateral mode of transmission for these symbionts, although the specific mechanism of transmission (i.e., host-host transfer or environmental acquisition) requires further study. Finally, the stable isotopes measured in this study indicate both thyasirids (*Parathyasira* sp., and *T.* aff. *gouldi*) may base their diet more on chemoautotrophic than photoautotrophic sources, confirming the importance of symbionts for nutrition in these species.

Introduction

The discovery of the giant tubeworm *Riftia pachyptila* at hydrothermal vents in the late 1970s led to worldwide interest in symbiotic, nutritional relationships between marine heterotrophic invertebrates and chemoautotrophic bacteria (i.e., chemosymbiosis, see review by Dubilier *et al.*, 2008). Chemosymbiotic associations are not limited to the deep sea and commonly occur in shallow, sulphidic marine sediments (Fisher, 1990; Dubilier *et al.*, 2008). Host species may associate with one or several symbiont phylotypes, although a single phylotype dominates within each host individual (e.g., Distel *et al.*, 1988). The host specificity of symbionts and the extent of host-symbiont co-speciation vary extensively among chemosymbiotic organisms, providing diverse insights on the evolution of these systems (Childress & Fisher, 1992; Fisher *et al.*, 1993; Gros *et al.*, 2003; Rouse *et al.*, 2004; Taylor & Glover, 2006; Nussbaumer *et al.*, 2006; Won *et al.*, 2008).

Of the six bivalve families known to be chemosymbiotic (Solemyidae, Mytilidae, Vesicomyidae, Lucinidae, Nucinellidea, and Thyasiridae - Fisher, 1990; Oliver & Taylor, 2012), the small, usually < 10 mm, thyasirid clams (SF Thyasiroidea, Taylor *et al.*, 2007a) are among the few containing both asymbiotic and symbiotic species. Thyasirid symbionts are extracellularly among the microvilli of gill epithelial cells (Southward, 1986; Dando & Spiro, 1986), whereas the chemosymbionts of most other bivalves, with the exception of certain bathymodiolids, occur intracellularly (for a list of symbiont locations in bivalves, see table 1 of Dubilier *et al.*, 2008). Symbiotic thyasirids vary

greatly in gill morphology. Some species possess highly modified gill filaments, characteristic of other chemosymbiotic bivalves, with distinct abfrontal thickening (i.e., 'type 3' gills: *Thyasira flexuosa, T. sarsi, & T. gouldi*, Dufour, 2005) whereas others possess simple, unmodified homorhabdic gill filament morphology (i.e., 'type 2' gills: *Thyasira (Parathyasira) equalis*, Dufour, 2005).

Symbiotic thyasirids periodically digest their symbionts to obtain nutrition in addition to that obtained via particulate feeding; lysosomal bodies and packed bacterial membrane whorls on transmission electron micrographs of bacteriocytes indicate symbiont digestion (Southward, 1986; Herry & Le Pennec, 1987; Dufour & Felbeck, 2006). Histoautoradiographic studies (Southward, 1986) and the depleted $\delta^{13}C$ signature of the clams' tissues (Spiro *et al.*, 1986; Dando & Spiro, 1993; Rodrigues & Duperron, 2011) demonstrate the contribution of symbiont-derived nutrition for thyasirids. Further, the degree of nutritional dependence on symbionts varies with environmental conditions such as particulate organic matter in suspension and sulphide concentrations (Dando & Spiro, 1993; Dufour & Felbeck, 2006). Thus, because thyasirids include both asymbiotic and symbiotic species, the latter possessing extracellular symbionts associated with variable gill morphologies and a fluctuating nutritional dependence on symbionts, they represent the most primitive stages of bivalve-bacteria symbiosis (Fisher, 1990).

Of the 90 plus living species of thyasirids described to date, the symbionts of only six species have been characterized: *Thyasira flexuosa* (Distel & Wood, 1992), *Conchocele sp.* (Imhoff *et al.*, 2003), *'Maorithyas' hadalis* (*Axinulus hadalis*) (Fujiwara *et al.*, 2001; Taylor *et al.*, 2007a), *Thyasira vulcolutre*, and two unknown species from the

Gulf of Guinea (Rodriguez & Duperron, 2011) and the Eastern Mediterranean (Brissac *et al.*, 2010). Based on the 16S rRNA, 23S rRNA and APS reductase gene sequences of these symbionts (Distel & Wood, 1992; Imhoff *et al.,* 2003; Rodriguez & Duperron, 2011), they belong to the gammaproteobacteria division. Similarly to the symbionts associated with the deep-sea vestimentiferan tube worm *Riftia pachyptila* (Felbeck *et al.*, 1981), symbionts associated with thyasirids are sulphur-oxidizing, as demonstrated by the activities of sulphur-oxidizing and carbon fixation enzymes (Dando & Southward, 1986; Herry & Le Pennec, 1987; Herry *et al.*, 1989).

Thyasirid hosts sampled from a variety of geographical locations associate with symbionts from distinct lineages, suggesting multiple symbiont acquisition events in the evolutionary history of the family (Distel & Wood, 1992; Imhoff *et al.*, 2003). Furthermore, symbionts of three species of thyasirids from the eastern Atlantic and Mediterranean Sea appear host-specific (Rodriguez & Duperron, 2011). Because geographical location might confound host-symbiont specificity interpretations (Nieberding *et al.,* 2008; Won *et al.,* 2008; Vrijenhoek, 2010), two or more co-occurring symbiotic thyasirid species should be investigated in order to better evaluate host-specificity in this group. Similarly, the mode of symbiont transmission (sensu Stewart *et al.*, 2008), either being vertical (i.e., symbionts passed from parent to offspring) versus lateral (i.e., symbionts acquired from the surrounding environment,) has not yet been demonstrated in thyasirids, most likely due to the low number of sequences obtained from symbionts associated with thyasirids. The specific mode used will have important consequences on the symbiont phylogeny in comparison to the hosts'; vertical

transmission acts to couple phylogenies (Peek *et al.*, 1998) because symbiont genomes are co-transmitted with host cytoplasmic genes (i.e., mitochondria), while lateral transmission decouples phylogenies (Vrijenhoek *et al.*, 2007), because free living symbionts can undergo recombination with other lineages in the external environment.

Although thyasirids are thought to represent the most primitive stages of bivalve-bacteria evolution, the host specificity of thyasirid symbionts remains unclear with no strong evidence for or against co-speciation in this group. We therefore construct phylogenetic trees of thyasirids and their symbionts to test for host specificity and co-speciation, using both published sequences and sequences obtained from thyasirid species collected from Bonne Bay, Newfoundland (Chapter 1, Chapter 2). One of the Bonne Bay species, *Thyasira (Parathyasira)* sp., is asymbiotic and distantly related to other thyasirids collected from Bonne Bay (Chapter1, Figure 1-4), and was initially used in the analysis due to the direct observation of what appeared to be bacteria; the amplification of 16S would then confirm whether these bacteria were closely related to other known symbiotic bacteria, or distantly related, suggesting they might be contaminants. The other thyasirids collected to date are closely related (>99.6% similarity), cryptic individuals affiliated with *Thyasira (Thyasira) gouldi* (Philippi, 1845) that could be broken down into two groups based on molecular markers (18S+28S) and gill filament morphology: the first group being symbiotic with abfrontally-expanded gills, 'type 3' gills (sensu, Dufour, 2005) and the second being asymbiotic with 'type 2' gills (sensu, Dufour, 2005; see Chapter 2).

In addition to molecular data, we used stable isotope ratios (e.g., $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) to determine the relative nutritional importance of chemosynthetically-derived (usually more ^{13}C-, ^{15}N-, and ^{34}S-depleted) and phototrophically-derived (more ^{13}C-, ^{15}N-, and ^{34}S-enriched - Paull *et al.*, 1985; Brooks *et al.*, 1987; Kennicutt *et al.*, 1992; Vetter & Fry, 1998; Levin & Michener, 2002; Carlier *et al.*, 2010) sources obtained by thyasirids in Bonne Bay.

Materials and Methods

Sampling site

We sampled thyasirids from Bonne Bay, a subarctic fjord on the west coast of Newfoundland, Canada (**Figure 3-1**: A), separated from the Gulf of St. Lawrence by a 50 m deep sill. The bay consists of two "arms", the South Arm (max depth ~100 m) and the East Arm (max depth ~ 230 m) (Conan *et al.*, 1996, Quijón & Snelgrove, 2005), the latter is further separated by a shallow (15 m depth) sill (**Figure 3-1**: B). Although sampling was conducted at several sites, including South Arm, we obtained thyasirids only from three sits in East Arm: Southeast Arm (S, 49°27'51.46"N, 57°43'09.04"W, 30 m depth), Deer Arm (D, 49°32'43.48"N, 57°50'28.45"W, 30 m depth), and Neddy's Harbour (N, 49°31'21.44"N, 57°52'11.07"W, 15 m depth).

Thyasirid collection

In total, we collected 7 *Thyasira (Parathyasira)* sp. and 29 *T.* aff. *gouldi* specimens using a Peterson grab (radius = 10.5 cm, height = 30 cm, volume ~ 2.08×104 cm^2) on three separate occasions: October 2009 (O-09), May 2010 (M-10), and August 2010 (A-10). We sieved sediments on a 1 mm mesh and separated thyasirid species based on shell characters (Oliver & Killeen, 2002) and molecular data.

DNA extraction, PCR amplification and sequencing

Upon collection, we carefully removed the thyasirid gills and placed them individually in vials of 95% ethanol. We extracted and purified DNA from the gill tissue using the QIAgen DNeasy® Blood and Tissue kit, following the spin-column protocol for animal tissues. We performed polymerase chain reactions (PCRs) using four sets of primers: 1) 18S [forward] – GCC AGT AGC ATA TGC TTG TCT C and 18S [reverse] AGA CTT GCC TCC AAT GGA TCC (Holland *et al.*, 1991), ~550 bp of host 18S nuclear rRNA; 2) 18S-5' [forward] CTG GTT GAT (C/T)CT GCC AGT (Winnepenninckx *et al.*, 1998) and 18S1100R [reverse] CTT CGA ACC TCT GAC TTT CG (Williams *et al.*, 2003), ~1000 bp of host 18S nuclear rRNA; 3) LSU-5' [forward] TAG GTC GAC CCG CTG AA(C/T) TTA AGC A (Littlewood *et al.*, 2000) and LSU1600R [reverse] – AGC GCC ATC CAT TTT CAG G (Williams *et al.*, 2003), ~1500 bp of host 28S nuclear rRNA; and 4) 27f [forward] AGA GTT TGA TCC TGG CTC AG and 1492r [reverse] GGT TAC CTT GTT ACG ACT T (Lane, 1991), ~1500 bp of bacterial 16S rRNA. We chose to investigate the 18S and 28S gene fragments because

they correspond to the majority of published thyasirid sequences (~16). For all three genes (18S, 28S, 16S), the presence of both variable and highly conserved regions makes these rRNA genes useful in phylogenetic studies at various systematic scales (Steiner & Hammer, 2000).

Each 25 µL PCR reaction used the Promega PCR Master Mix (Promega Corp.) containing 50 µ/mL Taq DNA polymerase, 400 µM of each dNTPs, reaction buffer at a pH of 8.5, and a gene specific concentration of $MgCl_2$: 3.0, 2.5, and 3.0 mM for 18S, 28S, and 16S, respectively. We set thermocycler conditions to: initial denaturation at 94 ^{0}C for 4 min, 35 cycles of denaturation at 94 ^{0}C for 30 sec, gene specific annealing temperature of 54, 52, and 50 ^{0}C for 18S, 28S, and 16S, respectively, and elongation at 72 ^{0}C for 2 min, with a final elongation at 72 ^{0}C for 5 min. We then filtered all amplified products using Acro-Pro® 100K-Omega filters (Pall Life Sciences), performed sequencing reactions using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems), and electrophoresed on an Applied Biosystems 3730 DNA Analyzer automated capillary sequencer running Sequencing Analysis v. 5.2 Software (Applied Biosystems).

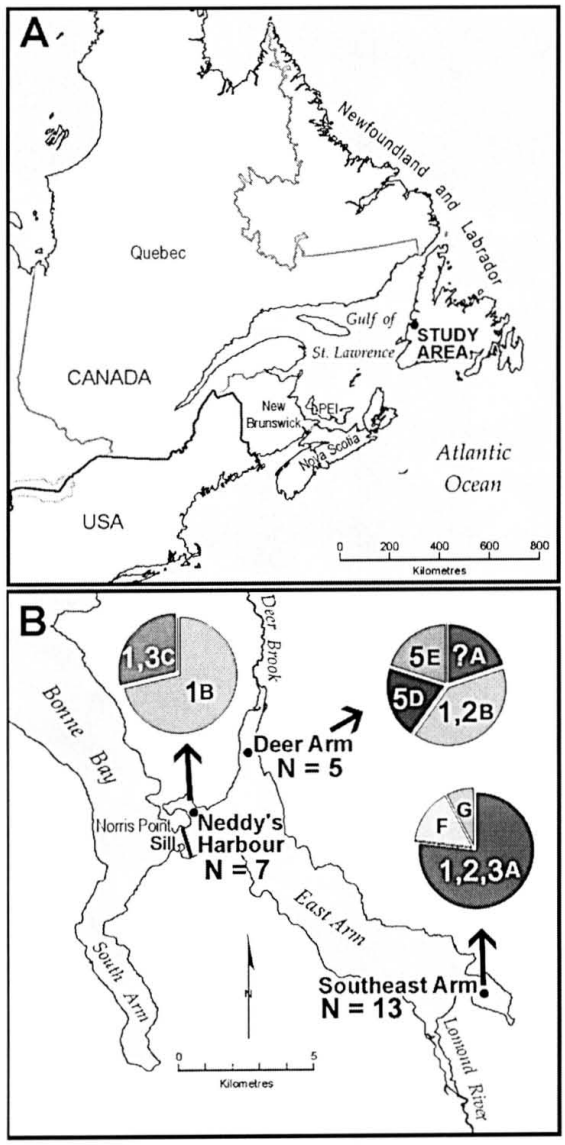

Figure 3-1: Map of Bonne Bay, Newfoundland. A. Study area (closed dot) in Newfoundland, Canada. B. Study area (Bonne Bay) with collection locations given: Deer Arm, Neddy's Harbour, and Southeast Arm. The pie charts at each location show relative proportions of OTUs (1-7), bacterial phylotypes (A-E), and total number of individuals (N) used in molecular analyses. The "?" indicates an individual which data was not available to identify host OTU. F and G are bacterial phylotypes associated with the asymbiotic *Parathyasira* sp (see Chapter 1).

Phylogenetic analyses

We used Sequencher® (v. 5.0, Gene Codes Corp.) to align both the forward and reverse sequences and then used the online Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990) to match sequences to those most closely related in GenBank (**Table 3-1**). Bacterial phylotypes were identified by aligning individual 16S sequences and grouping them based on polymorphic nucleotide sites; thus, phylotypes consisted of sequences containing no base discrepancies. We executed all alignments with default values in MEGA5 (Tamura *et al.*, 2011), using ClustalW (Thompson *et al.*, 1994). Similarly, we identified operational taxonomic units (OTUs) for 18S and 28S genes (Chapter 2).

We determined the relatedness between 16S phylotypes by calculating the average evolutionary percent divergence (%*d*) and constructing a distance matrix using "p-distance" as the evolutionary model in MEGA5; thus, we could compare the proportion of base discrepancies, represented as a percentage (% divergence), between pairs of phylotype regardless of fragment size. Similarly, we calculated pairwise genetic distances for bacterial 16S sequences using the Kimura 2-parameter (a.k.a K-2-P, Kimura, 1980) model, and the concatenated 18S+28S thyasirid sequences, using the Tajima-Nei (1984) model. These models were chosen based on the maximum likelihood test in MEGA5 within the "model selection" analysis.

We tested for correlation between host thyasirids and symbiont bacteria using PARAFIT (Legendre *et al.*, 2002), a software program that tests the hypothesis of co-speciation between a clade of hosts and a clade of associated symbionts. The null

hypothesis (H_0) of the overall test assumes independence of host and symbiont phylogenies, represented in two genetic distance matrices, but also test for correlation at each individual host-symbiont (H-S) link, established in a separate matrix. Evolutionary distance, as computed in MEGA5, is a "nonmetric or semimetric quantity" which needs to be converted to principle components in order to apply a linear ANOVA model (Legendre & Anderson, 1999). We converted the two distance matrices to principle components using DistPCoA (Legendre & Gallagher, 2001), and then used PARAFIT to assess the correlation between principle components.

Phylogenetic reconstruction

We constructed three phylogenetic trees. The first was based on the 16S gene, and included published thyasirid symbiont sequences, the bacterial phylotypes identified in this study, and other closely related free living bacteria. For the second and third trees, we used individual sequences (thyasirid concatenated 18S + 28S and corresponding bacterial 16S) obtained in this study (i.e., non-consensus sequences) to visualize host-specificity and test for correlation between thyasirids and their associated bacterial symbionts. All alignments used ClustalW in MEGA5, as previously described, in which we removed poorly aligned and ambiguous sites using the software program Gblocks (v. 0.91b, Castresana, 2000).

We estimated the tree of concatenated 18S+28S sequences using Bayesian methods (MrBayes, v 3.1.2, Huelsenbeck & Ronquist, 2001) so we could partition genes to apply specific models for each. We chose K-2-P for 18S and General Time Reversible

(GTR hereafter, Lanave *et al.*, 1984; Tavaré, 1986) for 28S using the Maximum-Likelihood statistical method in MEGA5 (Nei & Kumar, 2000). For this tree, we estimated base frequencies and set rate variation to gamma-distribution with four discrete categories and allowance for invariant sites. Data sets were run twice, with 1,000,000 generations each and a sampling frequency of once every 100 generations (both trees), basing the consensus tree on the combination of the trees remaining from each run (total =15.000). Support values associated with the nodes of both trees used posterior probabilities (PP) calculated in MrBayes.

We constructed the two bacterial 16S trees using Maximum Likelihood method in MEGA5 and determined K-2-P was the best substitution model, with allowance for invariant sites (16S sequences from this study only) and evolutionary rates gamma-distributed (16S phylotypes from this study with other bacterial sequences), as determined in the model test described previously. We based support values on 500 bootstrap replicates.

Stable isotope ratios

We obtained stable isotope ratios of carbon ($^{\delta 13}$C), nitrogen ($^{\delta 15}$N), and sulphur ($^{\delta 34}$S) ratios from tissue samples using an elemental analysis – isotope ratio mass spectrometer (EA-IRMS). We dissected 75 thyasirids, placed either gills or pooled foot and mantle tissues inside ultra-light aluminum (sulphur) or silver (carbon and nitrogen) capsules, decarbonated the latter samples by acid-fumigation with 6M HCl for 12 hours, and left them in a drying oven at 40 ^{0}C overnight. Gill and foot/mantle tissues were

separated so we could estimate the isotopic signature of symbionts (in the gills of symbiotic species but not in the foot and mantle). In addition to thyasirids, we analyzed tissues from two suspension-feeding bivalves using the same method as described above except that we pooled all tissues.

The samples were analyzed against the conventional standards: Pee Dee Belemnite (PDB) for carbon, atmospheric N_2 for nitrogen, and Canyon Diablo Triolite for sulphur, using the mass spectrometer available at the CREAIT-TERRA Stable Isotope facility at Memorial University, St. John's, Newfoundland. Because of the small body size of many of the thyasirids used in this analysis (<3 mm diameter and 0.300 mg dry weight), we had to pool tissues from more than one individual of a species in some cases. This was done prior to discovering that not all *T.* aff. *gouldi* were symbiotic, so pooled samples may include a combination of symbiotic and asymbiotic *T.* aff. *gouldi*, and is described further in the discussion.

Statistical analysis for isotope ratios

We used three general linear models (GLMs, one for each isotope) to detect whether stable isotope ratios were significantly different between samples, including the main effects of species (*T.* aff. *gouldi* vs. *T. (Parathyasira)* sp.), tissues (gills vs. foot/mantle), and the interactive effects of species and tissues. The values obtained from the suspension feeding bivalves were used as a general reference, and were not included in the statistical analysis. All analyses met the main assumptions of the residuals without any major violations (i.e., normal, homogenous, and independent residuals observed).

Results

Bacterial phylotypes and geographic distribution

The alignment containing bacterial16S sequences obtained from individuals in this study (N=25), included 1439 bp, in which 481 bp were variable sites. Based on these variable sites, we defined seven distinct phylotypes (A-G), the first five (A-E) associated with *Thyasira* aff. *gouldi*, and the last two (F, G) associated with *T. (Parathyasira) sp.* Average evolutionary percent divergence ranged from 0.3 to 24.5% (**Table 3-2**); phylotypes A-C were relatively similar (range: 0.9 – 1.2%), as were phylotypes F & G (0.3%) while the latter phylotypes were most distantly related to phylotypes D & E (range: 23.5 - 24.5%) (**Table 3-2**). Bacterial phylotypes appeared somewhat site-specific: phylotype A dominated Southeast Arm, and only C occurred at Neddy's Harbour (**Figure 3-1**: B). However, phylotypes A and B spanned more than one site: B in Neddy's Harbour (N=5) and Deer Arm (N=2), and A in Southeast Arm (N=11) and Deer Arm (N=1) (**Figure 3-1**: B).

Bacterial phylogeny

The reconstructed phylogeny based on the 16S bacterial phylotypes obtained in this study, other previously-published thyasirid symbionts (**Table 3-1**), and free-living bacteria (**Figure 3-2**) grouped phylotypes A-C together strongly (100% support value) with *T. flexuosa* symbionts from Plymouth Sound, England (accession # L01575) and from the Eastern Mediterranean (accession # FN600359). This group nested within

another strongly supported clade (100%) containing gammaproteobacteria, including all other confirmed thyasirid symbionts (*Maorithyas hadalis* symbionts I & II, *Conchocele disjuncta* symbiont, *T. vulcolutre* symbiont, *Thiobacilus thyasiris*), and a free-living bacterium isolated from Arctic sediment samples (accession # EU050850). Phylotype D also positioned with the gammaproteobacteria and related closely to a bacterium associated with the sea slug *Elysia ornata* (accession # AB196667, 98% identity). The strongly supported phylotype E group (100% support value) also contained epsilonproteobacteria, including a sequence extracted from *T. flexuosa* from the Eastern Mediterranean (accession # FN600361), and related most closely to *Sulfurimonas autotrophica* (accession # AB088432, 96% identity). Phylotypes F & G placed in a highly supported group (100% support value) that contained free-living deltaproteobacteria, and were most closely related to an uncultured bacterial isolate taken from ocean crust samples (accession # EU491252, 96% identity).

Table 3-1: Symbiont sequences associated with thyasirid hosts and other closely related sequences with collection locality, 16S fragment accession numbers, and respective references given. TBD, to be determined, FLB = free living bacteria.

Sample	Host/associated invert.	Locality	16S	Reference
FLB (raw seawater)	NA	Raw seawater	HQ203919	Bae & Lee, 2010
T. Parathyasira sp. Phylo. F	T. Parathyasira sp.	Bonne Bay, Newfoundland, Canada	TBD	Batstone et al., submitted
T. Parathyasira sp. Phylo. G	T. Parathyasira sp.		TBD	
Thyasira flexuosa sym. 2	Thyasira flexuosa	Eastern Mediterranean station 2A	FN600359	Brissac et al., 2010
Thyasira flexuosa sym. 1	Thyasira flexuosa		FN600361	
FLB clone (Arctic sed.)	NA	Arctic sediment	L01576	Distel & Wood, 1992
Thiobacillus thyasiris	NA	Plymouth Sound, England	L01479	
Thyasira flexuosa sym. 3	Thyasira flexuosa		L01575	
Maorithyas hadalis sym I	Maorithyas hadalis	Japan Trench	AB042413	Fujiwara et al., 2001
Maorithyas hadalis sym II	Maorithyas hadalis		AB042414	
Conchocele disjuncta sym.	Conchocele disjuncta	Sakhalin, Sea of Okhotsk, Trawl 25-1	AJ441190	Imhoff et al., 2003
Sulfurimonas autotrophica	NA	Hydrothermal seds., Mid-Okinawa Trough	AB088432	Inagaki et al., 2003
Endozoicomonas elysicola	Sea slug Elysia ornata	NA	NR_041264	Kurahashi & Yokota, 2006
Sulfurospirillum deleyianum	NA	Japan: Okinawa, Amami	AB368775	Matsumoto & Nishimura, 2007
FLB (hydro. chimney)	NA	Guaymas Basin, hydrothermal vent chimneys	DQ925889	Page et al., 2006
Helicobacter sp.	Muricea elongata	Environmental sample	DQ917867	Ranzer et al., 2006
T. vulcolutre sym.	T. vulcolutre	Gulf of Cadiz	FR715580	Rodrigues & Duperron, 2011
Thyasira sp. REGAB sym.	Thyasira sp. REGAB	Gulf of Guinea, Regab site	FR715579	
FLB (ocean crust)	NA	Ocean crust	EU491252	Santelli et al., 2008
FLB (hydro. sulphides)	NA	East Pacific Rise, hydrothermal sulphides	JQ287044	Sylvan et al., 2011
T. aff. gouldi Phylo. A	T. aff. gouldi (OTUs 1,2,3, ?)	Bonne Bay, Newfoundland, Canada	TBD	This study
T. aff. gouldi Phylo. B	T. aff. gouldi (OTUs 1&2)		TBD	
T. aff. gouldi Phylo. C	T. aff. gouldi (OTUs 1&3)		TBD	
T. aff. gouldi Phylo. D	T. aff. gouldi (OTU 5)		TBD	
T. aff. gouldi Phylo. E	T. aff. gouldi (OTU 5)		TBD	

Table 3-2: Genetic distance matrix. Based on bacterial 16S phylotypes (A-G), showing total no. of sequences (N Tot.), and no. of sequences (in brackets) at each site and associated with host OTUs 1-3 & 5 (*T.* aff. *gouldi*) and *T. (Parathyasira)* sp. Pairwise genetic distances calculated based on the Kimura-2-parameter (1980) method. Shading indicates degree of divergence, red being lowest and green highest. The "?" in the fourth column, second row refers to an individual in which 18S and 28S data was not available to confer OTU.

				Distance Matrix						
Phylotype	N Tot.	Sites	Host OTUs	A	B	C	D	E	F	G
A	11	S(10), D(1)	1(6), 2(3), 3(1), ?(1)	---						
B	7	N(5), D(2)	1(6), 2(1)	0.009	---					
C	2	N(2)	1(1), 3(1)	0.012	0.010	---				
D	1	D	5(1)	0.112	0.116	0.114	---			
E	1	D	5(1)	0.222	0.220	0.223	0.213	---		
F	2	S(2)	*T. (Parathyasira)* sp.	0.218	0.217	0.222	0.235	0.245	---	
G	1	S(1)	*T. (Parathyasira)* sp.	0.213	0.212	0.216	0.228	0.237	0.003	---

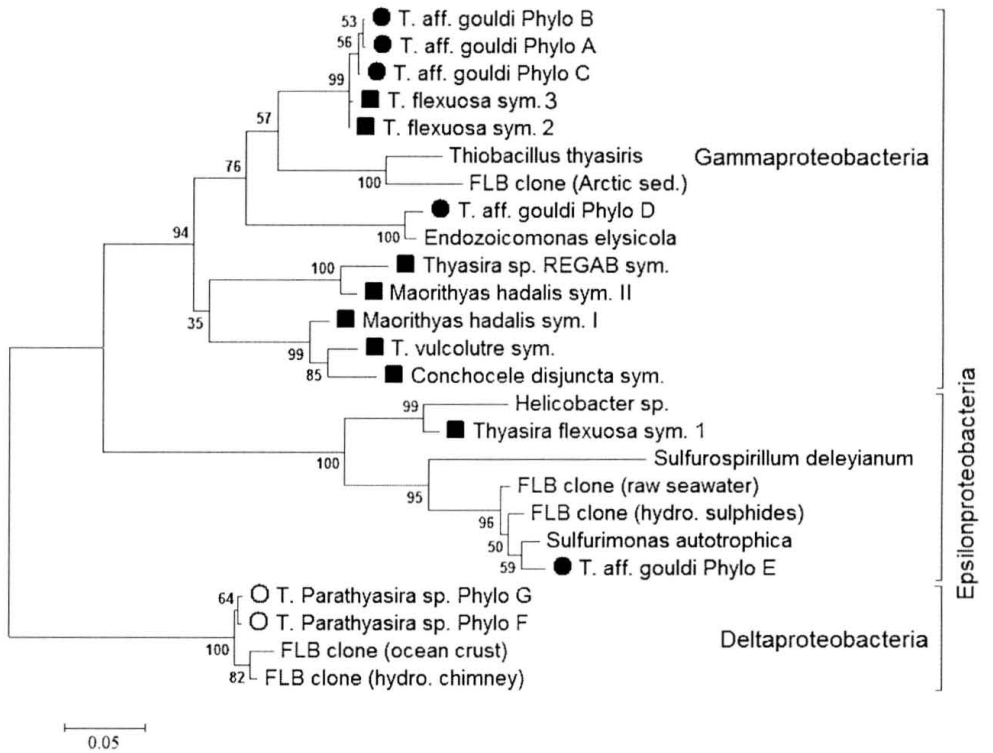

Figure 3-2: Un-rooted maximum Likelihood tree, featuring 16S bacterial phylotypes from this study (Phylo A-G) with previously published thyasirid symbionts and other free living bacteria (FLB). Evolutionary distances computed using Kimura-2-Parameter model, with Gamma-distribution to model evolutionary rate differences, and branch lengths measured in no. of substitutions per site. Open and closed circles show phylotypes associated with thyasirids from Bonne Bay while closed squares are associated with other thyasirids. See **Table 3-1** for description of sequences used. Evolutionary analyses were conducted in MEGA5.

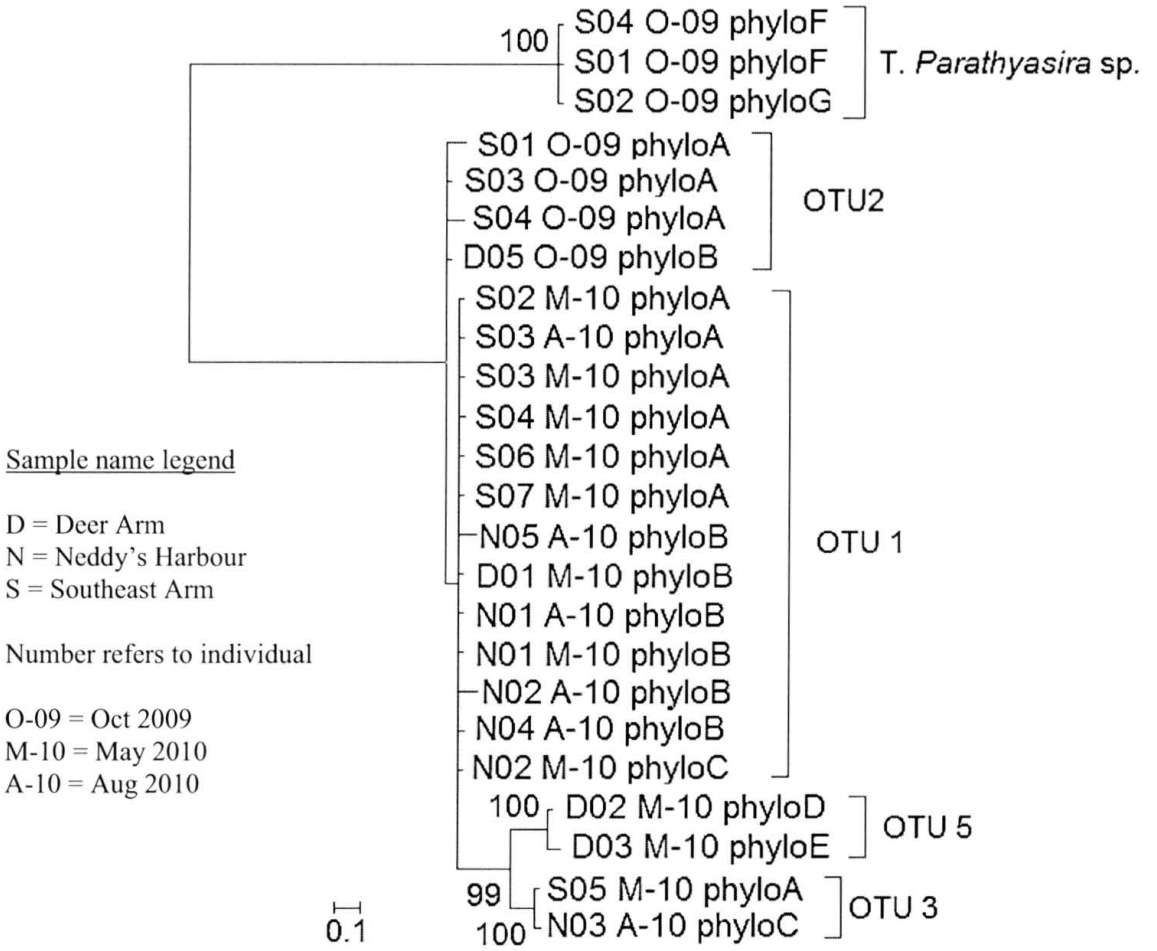

Figure 3-3: Bayesian tree, featuring thyasirid concatenated 18S + 28S sequences obtained in a previous study (Chapter 2). Evolutionary distances computed using Kimura-2-Parameter (18S) and General Time Reversible (28S) models, with branch lengths measured in no. of substitutions per site. Operational Taxonomic Units (OTUs) refer to *Thyasira* aff. *gouldi* only. Evolutionary analyses conducted in MrBayes (v3.1.2). Note that taxa do not correspond between this figure and **Figure 3-4**, so no attempt was made to connect hosts and symbionts.

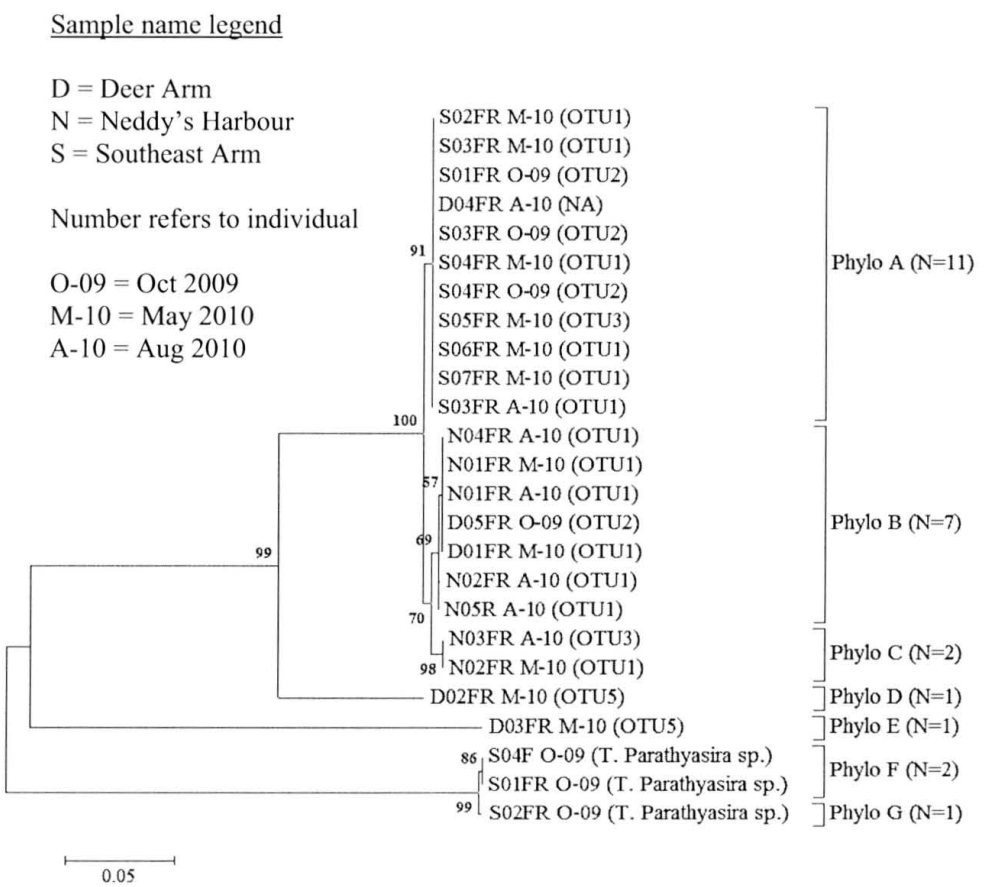

Figure 3-4: Un-rooted maximum Likelihood tree, featuring symbiont 16S sequences corresponding to host thyasirids obtained from this study. Evolutionary distances computed using Kimura-2-Parameter model, with allowance for invariant sites and branch lengths measured in no. of substitutions per site. Operational Taxonomic Units (OTUs) refer to *Thyasira* aff. *gouldi* hosts only. Evolutionary analyses conducted in MEGA5. Note that taxa do not correspond between this figure and **Figure 3-3**, so no attempt was made to connect hosts and symbionts.

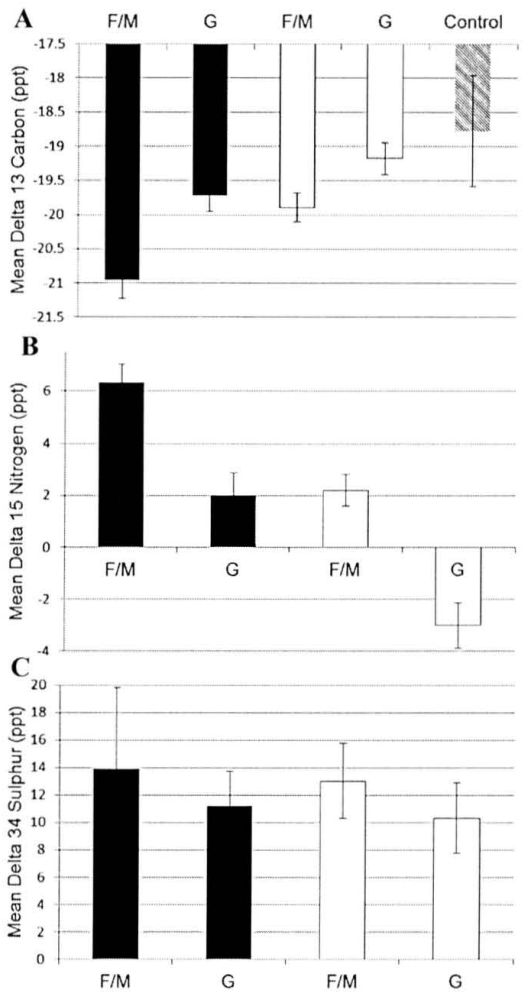

Figure 3-5: Average stable isotope values (A: $\delta^{13}C$; B: $\delta^{15}N$; C: $\delta^{34}S$). Foot and mantle (F/M) and gill (G) tissues of bivalve species (black bars = *T. (Parathyasira)* sp.; white bars = *T.* aff. *gouldi*; hatched bar = unknown heterotrophic control), with S.E. bars. No control species were available for nitrogen and sulphur isotopes.

Test of host-symbiont co-speciation

Comparing the thyasirid 18S+28S and corresponding 16S distance from this study (**Figure 3-3** & **Figure 3-4**) revealed independent host-symbiont phylogenies. For example, bacterial phylotype A associated with three thyasirid OTUs (1-3), phylotype B with two OTUs (1&2) and C with two OTUs (1&3), indicating that a single bacterial phylotype can associate with multiple host OTUs. We also observed the reverse situation, where a single host OTU associated with several bacterial phylotypes. For example, OTU1 associated with A-C, OTU2 associated with A&B, and OTU3 associated with A&C, indicating low host-symbiont specificity. Further, the PARAFIT test comparing thyasirid (18S+28S) and bacteria (16S) distances (**Table II,** Appendix) revealed no significant relationship ($p = 0.48$), indicating independent bacterial and corresponding thyasirid phylogenies.

Stable isotope analysis

The GLM conducted on $\delta^{13}C$ in thyasirid tissues revealed an insignificant interaction term between species and tissue [$F(1, 132) = 0.60$, $p = 0.44$]. Conversely, the main effects of this model revealed a significant ^{13}C-enrichment within gill tissues (mean ± SD = -19.39 ± 1.47‰) compared to foot and mantle tissues (-20.33 ± 1.37‰) for both *T.* aff. *gouldi* and *T. Parathyasira* sp. [$F(1, 132) = 16.62$, $p < 0.001$] (**Figure 3-5**: A). Further, *T.* aff. *gouldi* tissues were significantly more ^{13}C-enriched (-19.50 ± 1.46‰) than the tissues of *T. (Parathyasira) sp.* (-20.289 ± 1.431‰) [$F(1, 132) = 12.69$, $p < 0.001$] (**Figure 3-5**: A). The tissues from the two suspension-feeding bivalves were relatively

more ^{13}C-enriched (-18.78‰ ave.) than the thyasirids (-19.82 ± 1.49‰) (**Figure 3-5**: A); however, due to the low sample size for the control bivalves (N = 2), a statistical test was not possible to determine if this result was significant.

The GLM conducted on δ^{15}N (**Figure 3-5**: B) revealed an insignificant interaction term between species and tissue [$F(1, 83) = 0.65$, $p = 0.42$]. The main effects revealed significantly more ^{15}N-depletion within gill tissues (mean ± S.E. = -1.16 ± 5.05‰) than in foot and mantle tissues (mean ± SD = 3.689 ± 3.52), [$F(1, 83) = 25.22$, $p < 0.001$] and significantly greater ^{15}N-depletion in *T.* aff. *gouldi* (-0.68 ± 4.85‰) than in *T. (Parathyasira) sp.* (3.91 ± 3.95) [$F(1, 83) = 27.52$, $p < 0.001$]. Finally, the GLM on δ^{34}S was not significant for any effects [$F(3,12) = 0.25$, $p = 0.86$), the average value being +11.9‰ (SD=5.76) (**Figure 3-5**: C).

Discussion

Symbiont versus bacterial contaminant

Although we obtained seven distinct bacterial 16S phylotypes from thyasirids, only four belong to the gammaproteobacteria (A-D), the division in which all other thyasirid symbionts have been reported to date (**Figure 3-2**). The three most common bacterial phylotypes of this study (A,B,C; 80% of all 16S sequences obtained) associated with *Thyasira* aff. *gouldi* OTUs (1-3), previously determined to be symbiotic based on transmission electron micrographs (Chapter 2), and were genetically similar to one

another (<1.2% difference, **Table 3-2**). They were also most closely related to the previously-published *T. flexuosa* gill symbionts (Distel & Wood, 1992; Brissac *et al.*, 2010), suggesting symbionts rather than contaminants were likely represented.

In contrast, we obtained phylotype D, which was also within the gammaproteobacteria, from a single asymbiotic thyasirid (D03 M-10, host OTU 5, Chapter 2) (**Figure 3-4**); further, phylotype D related most closely to a bacterium associated with sea slugs (Kurahashi & Yokota, 2006), as opposed to other thyasirid gill symbionts (**Figure 3-2**). Similarly, we amplified phylotypes E, F, and G from only a few, asymbiotic (*T.* aff. *gouldi* OTU5 and *T. (Parathyasira)* sp.) individuals (**Figure 3-4**, N=1 or 2) and none were gammaproteobacteria. Phylotype E grouped within the epsilonproteobacteria, including another *Thyasira flexuosa*-associated bacterium from the Eastern Mediterranean (Brissac *et al.*, 2010); however, these authors suggested this sequence likely did not belong to a symbiont because of the lack of fluorescence following in-situ hybridization (FISH) in the gill tissues of the thyasirid specimen. Phylotypes F & G grouped within the deltaproteobacteria, and were most closely related to free-living bacteria obtained from sediment samples. Gutless oligochaete worms (*Olavius algarvensis*, Dubilier *et al.*, 2001) contain sulphate-reducing deltaproteobacterial symbionts, but sequences from our study related only distantly to these symbionts. Phylotypes D-F likely represent bacterial contaminants (i.e., sediment dwelling bacteria trapped on the gill mucociliary layer, whose 16S sequences amplified because they were the dominant bacteria on these otherwise asymbiotic gills) rather than symbionts. Further, we amplified each of these sequences only once and from a single location (ex. phylotype

D at Deer Arm in May 2010, phylotypes F and G at Southeast Arm in October 2009), despite several collection periods. In contrast, we would expect to observe symbionts on multiple occasions and at more than one site (ex. $symB_{VII}$ in Stewart *et al.*, 2008). In situ hybridization could confirm whether these bacteria are symbionts or contaminants. The latter might be in such low abundance that that they escape detection, or they could be confined to the frontal zone of the gill filaments where particles become trapped during suspension feeding. Brissac *et al.* (2010) detected a few positive epsilonproteobacterial cells in several of their thyasirid specimens; however these were confined to the ciliated zone of the gills and interpreted as contaminants. Phylotypes A-C likely represent symbionts and merit further discussion.

Host-symbiont relationships in closely related thyasirids

This study is the first to report the presence of several bacterial phylotypes associated with closely-related, co-occurring thyasirids, allowing the assessment of host-specificity and correlation between symbionts and hosts. The degree of host-symbiont specificity between thyasirids and symbionts requires confirmation beyond the low number of specimens reported in previous studies. For example, four individuals of *Thyasira flexuosa* collected from a single site (2A at 1693 m depth) in the Eastern Mediterranean associated with a single bacterial 16S phylotype (Brissac *et al.*, 2010); however, the authors suggested the need for more data to test whether sibling thyasirid species share the same symbionts, or if a host species associates with the same symbiont phylotype across its range.

Our sample size (N=25) is larger compared to previous work, and includes co-occurring thyasirids. We note a lack of host-symbiont specificity in our samples because different thyasirid OTUs share symbiont phylotypes (e.g., host OTUs 1, 2, & 3 share bacterial phylotype A at Southeast Arm) and a single host OTU associates with different phylotypes depending on the site (e.g., OTU 1 with phylotype A at Southeast Arm, B at Deer Arm, and B or C at Neddy's Harbour) (**Figure 3-1**: B & **Figure 3-4**). These results suggest that symbionts may be site-specific, a finding especially apparent at Neddy's Harbour where only symbiont phylotype C occurred (**Figure 3-1**: B).

The environmental infection model for vestimentiferan tube worms at hydrothermal vents (Nussbaumer *et al.*, 2006) proposes that the same local symbiont phylotype should infect different host species settling within the same habitat (also see Vrijenhoek, 2010). Assuming similar environmental symbiont acquisition in thyasirids, we infer some connectivity in symbiont populations between sampling sites: a single host OTU acquired the same phylotype from different sites (OTU 1 with B at both Neddy's Harbour [N=5] and Deer Arm [N=2]). We would anticipate such connectivity given the short distance between sites (<14 km) and similarity in habitats (glacial sediments within a subarctic fjord). Phylotype C, in contrast, may be restricted to Neddy's Harbour (based on sampling thus far, N = 7). Neddy's Harbour shallower (~15 m) depth contributes to greater temperatures and salinity fluctuations than the deeper Deer Arm and Southeast Arm (Chapter 1). Neddy's Harbour has also been subjected to anthropogenic activities including dredging (R. Hooper, pers. comm.), and contains coarser sediments than at the other two sites (personal observation). These differences may explain the unique

phylotype C at Neddy's Harbour, also associated with the rare host OTU 3 (N=2) (**Figure 3-1**, **Figure 3-4**).

We also determined that a single OTU could acquire more than one symbiont phylotype at a given site (OTU 1 at Neddy's Harbour with phylotype B [N=5] and phylotype C [N=2]), possibly as a result of the symbiont transmission mode in these thyasirids. For example, if bacteria are strictly acquired from a free-living local pool, microscale patchiness within a site may result in thyasirids acquiring different phylotypes on a small spatial scale. The high degree of patchiness in dissolved free sulphide and organic matter in the study sites supports this interpretation (Chapter 1).

Symbionts might also be transmitted horizontally between different host OTUs (i.e., horizontal transmission). Host-to-host transfer of the same symbiont lineage has been observed in other co-occurring invertebrate species (ex. *Drosophila ambigua* and *D. tristis*), which was attributed to these host species sharing the same food substrates or parasites (Haine *et al.*, 2005). In Bonne Bay thyasirids, symbionts released from one host species could potentially infect individuals of other host species nearby. We often collected multiple host OTUs within the same grab (OTUs 1 & 3 at Neddy's), potentially enabling the transmission of symbionts between hosts. Further, horizontal transmission is expected to have evolutionary consequences; the window of time for recombination to occur between thyasirid symbionts and the free-living bacterial pool would be reduced if symbionts are transferred directly from host to host. Thus, based on the principles of Muller's ratchet (Moran, 1996), we would expect genetic drift of the symbiont lineage to increase, possibly resulting in a relatively divergent phylotype, for example, Phylo. C in

Neddy's Harbour. Experimentation is necessary to confirm whether symbionts transfer from host-to-host (i.e., horizontal transmission), or if they are strictly acquired from the environment (i.e., environmental transmission). It would also be interesting to test whether these two types of lateral transmission produce different evolutionary consequences on the symbiont phylogeny.

The detection of host-symbiont coupling may be confounded by the host taxa used in the analysis (Page, 2003; Stewart *et al.*, 2008). We examined symbionts associated with thyasirid closely related OTUs (>99.5% identity), whereas previous studies investigated a single thyasirid species (Brissac *et al.*, 2010) or distantly related species (>2% divergence in 18S and 28S), which found distinct lineages of symbionts associated with different hosts (Rodrigues & Duperron, 2011). Conversely, our samples demonstrate low level host-symbiont specificity among closely-related host OTUs, because multiple symbiont lineages were found to associate with different hosts, and vise-versa.

Stable isotope analyses – preliminary findings

We conducted stable isotope analyses to determine whether bacteria contribute significantly to the thyasirid host diet. The $\delta^{13}C$ of the chemosymbiotic species *T.* aff. *gouldi* (-19.50‰) showed an unexpectedly greater ^{13}C-enrichment than other symbiotic thyasirids (e.g., *Thyasira sarsi*: -28.2 to -39.5‰, Spiro *et al*, 1986). The discrepancy reported here is most likely related to the specific form of RuBisCO present, which will have consequences on how carbon fractionates isotopically, thus, leading to differences in $\delta^{13}C$ (Robinson & Cavanaugh, 1995: Elsaied & Naganuma, 2001). Specifically, RuBisCO

forms I and II discriminate differently against ^{13}C, resulting in significant ^{13}C-depletion in organisms with RuBisCO form I (-27 to -35‰) whereas organisms with form II are more ^{13}C-enriched (-9 to -15 ‰) (Childress & Fisher, 1992; Robinson & Cavanaugh, 1995). We successfully amplified the *cbbM* gene associated with RuBisCO form II in symbiotic *T.* aff. *gouldi* from Bonne Bay (data not shown), but observed no amplification of the *cbbL* gene of form I (usually found in chemoautotrophic endosymbionts of deep-sea molluscs, Robinson & Cavanaugh, 1995). Therefore, given the chemoautotrophic bacteria associated with these thyasirids occur in the gills and possess form II RuBisCO, we would expect ^{13}C enrichment in the gills (compared to the foot and mantle) of those specimens (Spiro *et al.*, 1986). *T.* aff. *gouldi* tissues were less enriched in ^{13}C (average $\delta^{13}C$: -19‰) than other bacteria with form II RuBisCO (average $\delta^{13}C$: -11‰). This discrepancy may be the result of pooling symbiotic and asymbiotic OTUs; the greater degree of ^{13}C-depletion in asymbiotic individuals would bring the overall mean delta of ^{13}C value lower than expected for symbiotic individuals. Additionally, mixed sources of carbon from particulate feeding and symbiont digestion likely contributed to these results. By determining the stable isotope ratios of particulate organic matter (POM) in the water column and sediment, we could apply a mixing model (e.g., Conway *et al.*, 1989; Carlier *et al.*, 2010) that would specify the percentage of nutrition from each source.

We unexpectedly found that carbon and nitrogen isotope ratios both differed significantly between the asymbiotic gills of *T. (Parathyasira)* sp. and the foot and mantle tissues (**Figure 3-5**: A, B). Previous studies reported such differences in stable isotope ratios between gill and foot/mantle tissues in other asymbiotic bivalves (Spiro *et al.*,

1986; Conway *et al.*, 1989), and result from extensive lipid membranes in the gills where lipids are generally more ^{13}C-depleted (Conway *et al.*, 1989). However, relatively ^{13}C-enriched gills in an asymbiotic bivalve are surprising, and require further investigation.

Previous studies of the stable isotopic composition of thyasirids have found that *Thyasira sarsi*, known to be a chemosymbiotic species, exhibited significantly ^{13}C depleted tissues (δ^{13}C: -31.4 to -35.1‰) compared to tissues of other benthic fauna from the same region (-16 to -20 ‰), indicating that the source of carbon assimilated was mostly derived from bacteria utilizing reduced sulphur (Dando *et al.*, 1991). In contrast, δ^{13}C obtained from *T. equalis*, thought to be only partially chemosymbiotic, were relatively more ^{13}C-enriched, indicating that chemoautotrophic bacteria contributed less to its diet (Dando & Spiro, 1993). However, δ^{13}C values of both thyasirid species were more negative than those of other benthic fauna that were presumably heterotrophic and feeding primarily on phytoplankton (Dando & Spiro, 1993). Similarly, the δ^{13}C of heterotrophic bivalves used as a control in this study (-18.8‰) were within the range expected for organisms assimilating strictly photosynthetically-based nutritional sources (-16 to -20‰), and were slightly more ^{13}C-enriched compared to the thyasirids in our study, suggesting that both *T. (Parathyasira) sp.* and *T.* aff. *gouldi* assimilate chemoautotrophically-fixed as well as photoautotrophically-fixed carbon. For the asymbiotic species, the digestion of chemoautotrophic bacteria present in the sediment in addition to filtering out photoautotrophic sources from the water column may be potential mechanisms leading to a mixed carbon signature, while the symbiotic species digests the symbionts present in their gill in addition to suspension feeding.

Nitrogen isotope ratios of chemoautotrophic bacteria differ from those of photoautotrophic organisms (Lee & Childress, 1994), usually because of differences in nitrogen assimilation enzymes (e.g., in the case of ammonium: glutamine synthetase and glutamate dehydrogenase). Specifically, when nitrogen sources are non-limiting, chemoautotrophically-assimilated nitrogen is often ^{15}N-depleted (Rau, 1981a; Brooks *et al.*, 1987; Conway *et al.*, 1989; Fisher, 1990; Kennicutt *et al.*, 1992; Lee & Childress, 1994). The nitrogen sources used by three chemosymbiotic organisms (*Solemya reidi* from a sewage outfall, *Riftia pachyptila* from a deep sea vent, and an undescribed mussel species from a deep sea seep) differed at each site, and the assimilation rate of a particular nitrogen source by the symbiont-containing tissues of the host reflected the site-specific concentrations of each source (Lee & Childress, 1994). For example, the sewage outfall habitat exhibited high concentrations of ammonium compared to nitrate, thus, the assimilation rate of ammonium outweighed that of nitrate (Lee & Childress, 1994). However, the asymbiotic species *Mytilus edulis* did not assimilate inorganic nitrogen, obtaining its nitrogen from an alternative source not measured in the study. Differences in nitrogen sources at each site (combined sources vs. ammonium or nitrate), as well as the availability of the source (limited vs. unlimited) can affect the ^{15}N ratios of chemosymbiotic organisms.

Although we did not investigate the potential sources of nitrogen in this study, we obtained significant differences in nitrogen isotope ratios (δ^{15}N) for *T.* aff. *gouldi* (more ^{15}N-depleted) and *T. Parathyasira* sp. (more ^{15}N-enriched) (**Figure 3-5**: B), suggesting the two species use different, or more or less available, nitrogen sources. Further, the

range of $\delta^{15}N$ obtained here (-11.1 to +8.9‰) corresponded to values for chemoautotrophic bacteria (-13 to +5‰, Brooks *et al.*, 1987; Conway *et al.*, 1989; Fisher, 1990; Kennicutt *et al.*, 1992; Rau, 1981b). A significant proportion of the diet of the asymbiotic *T. Parathyasira* sp. may be chemosynthetically-derived; this species may feed mainly on bacteria in the sediment as opposed to POM in the water column. This suggests that symbiosis in thyasirids is opportunistic; for example, if particulate food availability is low, having symbionts would be advantageous for thyasirids because they would have an alternative food source available to them. However, if particulate food availability is relatively high, or if the pool of free-living chemoautotrophic bacteria in the sediment is large, then symbiosis might not provide an evolutionary advantage, and therefore, symbiosis may not be established. In addition, we found the expected pattern of ^{15}N-depletion in gill tissues compared to foot/mantle tissues (**Figure 3-5**: B) in *T.* aff. *gouldi*, and, unexpectedly, in *T. (Parathyasira)* sp., possibly for the same reasons as for carbon (composition of gill tissues).

The stable sulphur isotopes obtained here did not differ significantly between the two thyasirid species or between tissues; however, the overall average (+11.9‰) was slightly more ^{34}S-depleted compared to the range expected for POM in the water column (+17 to +21‰, Peterson & Fry, 1987) and slightly more ^{34}S-enriched compared to the range expected for sulphur-oxidizing bacteria (-25 to +5‰, Carlier *et al.*, 2010), suggesting a possible mix of sulphur sources. Again, obtaining sulphur isotopes for sediment and POM in the water column might shed light on the results obtained here.

In summary, as a result of the occasional pooling of thyasirids, and perhaps low sample size (N=16 for sulphur versus N=134 for carbon), we consider stable isotope results preliminary. Further investigation is needed to obtain C, N and S isotopes for POM and sediment samples and apply two-source mixing models. The form of RuBisCO impacts carbon isotope ratios, and should lead to ^{13}C enrichment in the gills of the more symbiotic species. Stable isotope ratios can greatly increase knowledge of the relationship between thyasirids and symbionts, and should be implemented in future studies.

Conclusion

The cryptic, closely related symbiotic thyasirids collected from the Bonne Bay fjord were found to lack specificity and co-speciation with their associated symbionts, based on comparing the reconstructed bacterial 16S and host 18S+28S phylogenies. Symbionts appeared to be slightly site-specific, although some phylotypes could be found at more than one site, most likely because of the close geographical proximity of sites (<14 km). Based on these findings, symbionts are inferred to be transmitted laterally, although whether they are strictly acquired from the free-living pool in the surrounding environment, or can be transmitted directly between hosts still needs to be confirmed. Further, the nutritional strategies of Bonne Bay thyasirids were investigated via stable isotope analyses, indicating both chemosynthetically-fixed and photoautotrophically-fixed sources contribute to the diets of both symbiotic and asymbiotic species, although differences were observed between species; symbiotic thyasirids exhibited a greater

degree of ^{13}C-enrichment and ^{15}N-depletion compared to asymbiotic species, suggesting a more chemosynthetically-based diet for the former compared to the latter. Additional investigation is required to shed more light on the nutritional strategies of these species, particularly by determining the stable isotope compositions in both POM in the water column and sediment and applying a two-source mixing model to identify the proportion of nutrition each source contributes to. Overall, a greater understanding of the symbiotic relationship between closely-related thyasirids and symbiotic bacteria is provided.

Thesis Summary

Results and conclusions

Thyasirid bivalves (Bivalvia: Thyasiroidea) have received little attention in the Northwest Atlantic compared to the Northeast Atlantic; as a result, the identity and dispersal potential of species across the Atlantic (amphi-Atlantic distribution) has not yet been determined. Based on several sampling trips in Bonne Bay fjord, on the west coast of Newfoundland, Canada, at least two different subgenera are present, *Thyasira (Parathyasira) sp.*, and *Thyasira (Thyasira) sp.*, based on several key morphological characteristics including shell shape, internal anatomy, and gill filament structure and molecular markers, including 18S and 28S rRNA nuclear gene fragments.

The first species, *Thyasira (Parathyasira)* sp., most closely resembles both *Thyasira (Parathyasira) equalis* (Verrill & Bush, 1898) and *T. (P.) dunbari* (Lubinsky, 1976), the former reported from the Gulf of Maine, and the latter from Canadian Arctic-Greenlandic region. However, *Parathyasira* sp. possesses distinct differences from these two species as well, suggesting it might be a new species; type material for *T. equalis* is needed to confirm this. The gill filaments of *Thyasira (Parathyasira)* sp. are not abfrontally-expanded, and bacterial symbionts or bacteriocytes were not observed, confirming it is an asymbiotic species. The second subgenera of thyasirids most closely resembled *Thyasira (Thyasira) gouldi* (Philippi, 1845) based on shell characteristics. This species has been reported in various locations around the Eastern Atlantic. However, the molecular markers (18S + 28S) obtained from these individuals revealed a high amount

of genetic variation, such that seven operational taxonomic units (OTUs) were defined. Upon examination of the gill filament morphology, OTUs 1-4 possessed abfrontally-expanded gill filaments in which bacteria were abundant in bacteriocytes, while OTUs 5-7 possessed simple gill filaments without abfrontal expansion, in which bacteria were never observed. Based on these striking differences, a species complex consisting of at least two sympatric species are suggested; speciation is proposed to have recently occurred within the Bonne Bay Fjord (i.e., since the last glacial maximum, ~ 20, 000 years BP). Such a large morphological and symbiosis-related disparity has never before been documented in species this closely related, suggesting that chemosymbiosis-related characters are important for setting up barriers of gene flow.

For the individuals confirmed as being symbiotic (i.e., *T.* aff. *gouldi* OTUs 1-4), three different symbiont phylotypes were shared among OTUs, suggesting a low host-specificity. Further, the dominant bacterial phylotypes were found to differ between collection sites, suggesting a greater site-specificity, although some instances of overlap were noted due to the close proximity of collection sites. The constructed phylogenies based on 18S and 28S rRNA fragments of host species and the corresponding 16S rRNA of bacteria were independent from one another, as demonstrated by the PARAFIT test, indicating that co-speciation is lacking. The mode of symbiont transmission for thyasirids in the Bonne Bay fjord is suggested to be lateral, although it is still unclear whether symbionts are transferred from individuals close in proximity (i.e., host-host horizontal transfer) or if they are strictly acquired from the surrounding environment (i.e., environmental acquisition).

Future directions and considerations

Research on thyasirids from Bonne Bay would benefit from the development of quick and easy techniques to identify *Thyasira* aff. *gouldi* OTUs. We could select restriction enzymes that could lead to informative restriction fragment length polymorphisms (RFLPs) in the variable regions of the 18S or 28S genes.

To strengthen phylogenetic analyses, it is important to amplify mitochondrial DNA (mtDNA) from thyasirids, particularly the Cytochrome Oxidase I (COI) gene, to determine whether the seven OTUs defined using nuclear genes also group in the same way with an additional gene marker. Further, mtDNA acquires nucleotide substitutions at a higher rate than nuclear DNA, making it useful for population genetic studies and for phylogenetic analysis of closely related taxa.

To confirm whether bacterial phylotypes associated with thyasirids in this study were symbionts as opposed to contaminants, florescence in-situ hybridization (FISH) could be used to bind phylotype-specific fluorescent probes with bacteria in the gill (if present), revealing the specific location of these bacteria. Also, the presence of more than one phylotype might be detected by such an approach.

Further stable isotope analyses (SIA) should take place to confirm whether thyasirids are deriving a significant proportion of their nutrition from symbionts. In conducting SIA, it is extremely important to know exactly which species (or OTU) is used; presently, the only way to confirm species (or OTU) identity and determine whether it is symbiotic or not (especially for the *T.* aff. *gouldi* species complex), is by sectioning the gills to observe gill morphology and performing PCR to identify variable sites that

correspond to a predetermined OTU. These analyses, however, require at least one gill, leaving too little material to conduct SIA (the dry weight of most thyasirid gills is <0.3 mg). If species (or OTUs) could be identified via shell morphology, then enough material should be available to conduct SIA. Fine-scale morphological differences in the shell might be discernible through shell shape analyses; such work is currently being done on to determine whether a significant difference in shell outline exists between OTUs.

Thyasirids are abundant in the Bonne Bay fjord and are expected to occur elsewhere around the province, especially in areas of moderate to high organic enrichment. By pairing morphological with molecular characteristics, thyasirids can be characterized with greater confidence. This research was the first to show the existence of species complexes in the thyasirid family, and that even closely related species can possess striking differences in terms of chemosymbiosis-associated morphology (i.e., gill filament structures). We also confirmed that host-specificity and co-speciation is lacking between thyasirids and symbiotic bacteria. The research conducted here can serve as a basis for understanding the evolution of thyasirids in organically enriched habitats.

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Appendix

Table I: Thyasirid species used in phylogenetic analysis, with collection locality, 18S + 28S accession numbers, and respective references given.

Host	Locality	18S	28S	References
Thyasira flexuosa MT-1	Eastern Mediterranean	FN600366	FN600362	(Brissac *et al.*, 2010)
Thyasira flexuosa MT-2	Eastern Mediterranean	FN600367	FN600363	
Thyasira flexuosa MT-3	Eastern Mediterranean	FN600368	FN600364	
Thyasira flexuosa MT-4	Eastern Mediterranean	FN600369	FN600365	
Thyasira vulcolutre	Gulf of Cadiz	FR716452	FR716453	(Rodrigues & Duperron, 2011)
Thyasira sp. REGAB	Gulf of Guinea, Regab site	FR716450	FR716451	
Axinulus sp.	Scotia Ridge, Antarctica	AM392441	AM392440	(Taylor *et al.*, 2007a)
Adontorhina cyclia	San Diego, California	AM392455	AM392438	
Leptaxinus indusarium	Arabian Sea, Pakistan	AM392454	---	
Thyasira methanophila	Concepción, Chile	AM392447	AM392431	
Thyasira perplicata	Angola	AM392448	AM392432	
Thyasira cf. subovata	Scotia Ridge, Antarctica	AM392451	AM392435	
Thyasira sp. (vent species)	Fiji Back Arc Basin	AM392452	AM392436	
Mendicula ferruginosa	Northern North Sea	AM774483	AM779657	(Taylor *et al.*, 2007b)
Parathyasira equalis	Gullmarsfjorden, Sweden	AM774482	AM779656	
Thyasira polygona	Northern North Sea	AM774484	AM779658	
Thyasira sarsi	Northern North Sea	AM774485	AM779659	
Thyasira gouldi	Mill Bay, Salcombe, UK	JF899224	JF899196	(Distel *et al.*, 2011)
Cardita leana	Hong Kong	AM774481	AM779655	(Taylor *et al.*, 2007b)
Eucrassatella cumingii	Moreton Bay, Queensland, Australia	AM774479	AM779653	

Table II: Pairwise genetic distance matrices. Based on bacterial 16S sequences (above diagonal) and corresponding 18S+28S thyasirid sequences (below diagonal) used in co-speciation test (Chapter 3). Outlined numbers indicate highest recorded distances (range: 0.214-0.319 in 16S, 0.048-0.056 in 18S+28S).

Sample		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
D01_M-10	1	---	0.127	**0.266**	0.000	0.000	0.000	0.002	0.011	0.011	0.000	0.000	**0.253**	0.010	**0.258**	0.009	0.008	0.010	0.010	**0.275**	0.010	0.010	0.010	0.007	0.010
D02_M-10	2	0.007	---	**0.252**	0.128	0.128	0.128	0.127	0.124	0.124	0.128	0.119	**0.270**	0.123	**0.273**	0.121	0.120	0.122	0.122	**0.298**	0.122	0.122	0.123	0.121	0.122
D03_M-10	3	0.007	0.000	---	**0.265**	**0.267**	**0.266**	**0.267**	**0.266**	**0.266**	**0.268**	**0.238**	**0.283**	**0.266**	**0.288**	**0.263**	**0.265**	**0.264**	**0.264**	**0.319**	**0.264**	**0.264**	**0.265**	**0.261**	**0.265**
D05_O-09	4	0.002	0.008	0.008	---	0.000	0.000	0.002	0.011	0.011	0.000	0.000	**0.254**	0.011	**0.258**	0.010	0.009	0.011	0.011	**0.275**	0.011	0.011	0.011	0.008	0.011
N01_A-10	5	0.000	0.007	0.007	0.002	---	0.000	0.002	0.011	0.011	0.000	0.000	**0.252**	0.011	**0.256**	0.010	0.009	0.011	0.011	**0.275**	0.011	0.011	0.011	0.008	0.011
N01_M-10	6	0.000	0.007	0.008	0.002	0.000	---	0.002	0.011	0.011	0.000	0.000	**0.254**	0.011	**0.258**	0.010	0.009	0.011	0.011	**0.276**	0.011	0.011	0.011	0.008	0.011
N02_A-10	7	0.000	0.008	0.008	0.002	0.000	0.000	---	0.010	0.010	0.002	0.000	**0.251**	0.011	**0.255**	0.011	0.010	0.011	0.011	**0.276**	0.011	0.011	0.011	0.008	0.011
N02_M-10	8	0.000	0.007	0.007	0.002	0.000	0.000	0.000	---	0.000	0.011	0.006	**0.254**	0.013	**0.257**	0.012	0.011	0.013	0.013	**0.278**	0.013	0.013	0.013	0.012	0.013
N03_A-10	9	0.006	0.004	0.005	0.004	0.006	0.006	0.007	0.006	---	0.011	0.006	**0.251**	0.012	**0.254**	0.012	0.011	0.012	0.012	**0.275**	0.012	0.012	0.012	0.012	0.012
N04_A-10	10	0.000	0.007	0.007	0.002	0.000	0.000	0.000	0.000	0.006	---	0.000	**0.249**	0.010	**0.253**	0.010	0.009	0.010	0.010	**0.274**	0.010	0.010	0.010	0.007	0.010
N05_A-10	11	0.000	0.006	0.006	0.002	0.000	0.000	0.000	0.000	0.002	0.000	---	**0.214**	0.003	**0.215**	0.003	0.002	0.003	0.003	**0.225**	0.003	0.003	0.003	0.003	0.003
S01_O-09_T.dun	12	**0.054**	**0.055**	**0.055**	0.017	**0.054**	**0.053**	**0.054**	**0.054**	**0.055**	**0.054**	0.017	---	**0.251**	0.004	**0.250**	**0.244**	**0.249**	**0.247**	0.000	**0.251**	**0.247**	**0.247**	**0.248**	**0.248**
S01_O-09_T.gou	13	0.001	0.008	0.008	0.000	0.001	0.001	0.001	0.001	0.007	0.001	0.002	**0.054**	---	**0.256**	0.000	0.000	0.000	0.000	**0.273**	0.000	0.000	0.000	0.000	0.000
S02_O-09_T.dun	14	**0.054**	**0.056**	**0.055**	0.017	**0.054**	**0.053**	**0.055**	**0.054**	**0.056**	**0.054**	0.019	0.000	**0.054**	---	**0.254**	**0.246**	**0.253**	**0.251**	0.003	**0.255**	**0.250**	**0.251**	**0.253**	**0.252**
S02_M-10	15	0.000	0.007	0.007	0.002	0.000	0.000	0.000	0.000	0.006	0.000	0.000	**0.054**	0.001	**0.054**	---	0.000	0.000	0.000	**0.272**	0.000	0.000	0.000	0.000	0.000
S03_A-10	16	0.000	0.007	0.007	0.002	0.000	0.000	0.000	0.000	0.006	0.000	0.000	**0.054**	0.001	**0.055**	0.000	---	0.000	0.000	**0.266**	0.000	0.000	0.000	0.000	0.000
S03_M-10	17	0.000	0.007	0.007	0.002	0.000	0.000	0.000	0.000	0.006	0.000	0.000	**0.054**	0.001	**0.054**	0.000	0.000	---	0.000	**0.271**	0.000	0.000	0.000	0.000	0.000
S03_O-09	18	0.002	0.008	0.008	0.000	0.002	0.002	0.002	0.002	0.004	0.002	0.002	0.017	0.000	0.017	0.002	0.002	0.002	---	**0.270**	0.000	0.000	0.000	0.000	0.000
S04_O-09_T.dun	19	**0.054**	**0.056**	**0.055**	0.017	**0.054**	**0.053**	**0.055**	**0.054**	**0.056**	**0.054**	0.019	0.000	**0.054**	0.000	**0.054**	**0.055**	**0.054**	0.017	---	**0.271**	**0.267**	**0.270**	**0.268**	**0.272**
S04_M-10	20	0.000	0.006	0.006	0.002	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.017	0.002	0.019	0.000	0.000	0.000	0.002	0.019	---	0.000	0.000	0.000	0.000
S04_O-09_T.gou	21	0.001	0.008	0.008	0.000	0.001	0.001	0.001	0.001	0.007	0.001	0.002	**0.054**	0.000	**0.054**	0.001	0.001	0.001	0.000	**0.054**	0.002	---	0.000	0.000	0.000
S05_M-10	22	0.006	0.004	0.005	0.004	0.006	0.006	0.007	0.006	0.000	0.006	0.002	**0.056**	0.007	**0.056**	0.006	0.006	0.006	0.004	**0.056**	0.002	0.007	---	0.000	0.000
S06_M-10	23	0.000	0.008	0.008	0.002	0.000	0.000	0.000	0.000	0.007	0.000	0.000	**0.048**	0.001	**0.048**	0.000	0.000	0.000	0.002	**0.048**	0.000	0.001	0.007	---	0.000
S07_M-10	24	0.000	0.007	0.007	0.002	0.000	0.000	0.000	0.000	0.006	0.000	0.000	**0.054**	0.001	**0.054**	0.000	0.000	0.000	0.002	**0.054**	0.000	0.001	0.006	0.000	---