SPIRORCHIID TREMATODES OF SEA TURTLES IN FLORIDA: ASSOCIATED DISEASE, DIVERSITY, AND LIFE CYCLE STUDIES

By

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To my family

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Abstract of Dissertation Presented to the Graduate School
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SPIRORCHIID TREMATODES OF SEA TURTLES IN FLORIDA: ASSOCIATED-DISEASE, DIVERSITY, AND LIFE CYCLE STUDIES

By

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Spirorchiid trematodes or blood flukes of sea turtles have been known to science for nearly 150 years. Like related parasites, such as schistosomes, of mammals, birds, and other taxa, spirorchiids primarily inhabit the vascular system where they cause injury to the host by damaging vessels, impeding circulation, and eliciting an inflammatory response. Many genera and species have been described from sea turtles, although their taxonomy has been complicated by multiple revisions, an inadequate specimen base, and incomplete descriptions. Likewise, studies of the effects of these parasites on sea turtle health have been limited in both numbers of hosts examined and geographical regions studied. There have been no large scale studies of spirorchiid trematodes of sea turtles of Florida, although these parasites have been a health issue of concern for many years. In addition, none of the life cycles have been discovered for any marine spirorchiid species or for any digenean trematode parasite of sea turtles, despite the rich diversity of Digenea in these animals. As a result, the emergent understanding of the spirorchiids and their impact on sea turtles is incomplete in many fundamental areas.

Our study investigated three different, but highly inter-related aspects of spirorchiids and their relationship with sea turtles in Florida including: characterization of associated disease in stranded loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) turtles; study of genetic

diversity and correlation with parasite microhabitat and host usage; and development and application of a molecular screening technique for life cycle discovery. Spirorchiids were found in high prevalence in stranded turtles and were associated with pathological lesions of concern in a significant number of animals. Differences in infection among size classes were identified, which have implications on disease studies and clinical management. Furthermore, we were able to demonstrate substantial previously unrecognized diversity among marine spirorchiids that correlated in many instances with microhabitat/host organ usage, and thus is relevant to associated disease and diagnosis. Lastly, the molecular tools and genetic database developed during this study were successfully applied to life cycle discovery efforts in the field and yielded the first evidence of intermediate host species utilized by sea turtle trematodes.

CHAPTER 1
INTRODUCTION

Background

Many aspects of sea turtle biology, ecology, health, and disease are poorly understood due to study limitations associated with the marine environment and the complex life histories. Ontogenic shifts and migrations occur over thousands of kilometers of ocean and are set in a diverse array of marine habitats. Life stages of sea turtles literally are distributed across the globe, often resulting in profound knowledge gaps. Thus, like many sea turtle studies, much of the information about diseases of these animals is obtained from land, where all turtles begin and a small number end their lives. A glimpse at health problems in wild sea turtles is obtained by examining stranded animals, which although a somewhat biased population and representing only a fraction of at-sea mortality, provide a wealth of information.

All sea turtle species currently are classified as endangered or threatened. Anthropogenic causes of population declines include over-harvesting, incidental take by various fisheries, destruction of nesting beaches, and deaths associated with other human activities. In addition, a number of diseases of sea turtles, including infectious diseases, various syndromes, and toxicoses, have been documented and some have been associated with mass mortality events. Of the reported infectious diseases, studies on fibropapillomatosis (FP) and parasites, especially spirorchiid trematodes or spirorchiids, comprise a large percentage of the sea turtle disease literature. Both are regarded as important health problems in wild turtles based on prevalence, associated pathology, and implications on survival.

Although well-recognized as pathogens of concern in sea turtles, spirorchiids had never been intensively studied in Florida turtles beyond initial descriptions and reports. In late 2000 and early 2001, an unusual mortality event characterized by neurological signs affecting *Caretta*

caretta occurred in south Florida (Jacobson et al., 2006). During this event, neurological infection by unidentified spirorchiids within the genus *Neospirorchis* was implicated as a possible contributing cause of neurological signs observed in the affected turtles. Several deficiencies in the general understanding of these parasites were identified or highlighted during the investigation of this event ranging from inability to specifically identify the parasites in many instances to the lack of knowledge of prevalence of parasites, intensities, and associated pathological lesions in Florida turtles. Furthermore, all of the marine spirorchiid life cycles, which have fundamental implications on the epidemiology and disease ecology of spirorchiidiasis, are unknown. The 2000/2001 *C. caretta* mortality event was a primary impetus for further study of spirorchiidiasis in Florida sea turtles.

Goals and Objectives

The aim of these studies was to gain a better understanding of spirorchiid trematodes in several key areas: 1) characterize spirorchiidiasis in stranded Florida sea turtles with regard to prevalence, associated pathology, and significance in terms of cause of death based on necropsy and parasitology findings; 2) investigate the potential for parasite diversity using molecular prospecting techniques in combination with observations in the turtle host and morphological studies; 3) develop a means of molecular identification and detection; and 4) apply these molecular techniques to life cycle discovery in field study sites. The first objective contributes to a basic tenant of population health assessment, which is to establish a pathologic database. This information is especially sparse for *Caretta caretta*. These objectives encompass a broad range of topics including basic pathology and disease surveillance, classical and molecular parasitology, and marine biology. Thus, a review of the literature includes relevant sea turtle biology, an in-depth review of spirorchiids and associated disease, and application of molecular techniques in parasitology studies.

Review of the Literature

Sea Turtle Biology: Florida Species Records, Life History, and Diet

Six species of sea turtle are found in Florida waters including the loggerhead (*Caretta caretta*), green turtle (*Chelonia mydas*), hawksbill turtle (*Eretmochelys imbricata*), Kemp's ridley (*Lepidochelys kempi*), olive ridley (*Lepidochelus olivacea*), and leatherback (*Dermochelys coriacea*). All sea turtles are classified as threatened or endangered and are protected under state and federal laws. The most frequently encountered species in Florida are *C. caretta* and *C. mydas*, which were the focus of these studies.

The life histories of sea turtles are complex and three major patterns have been characterized (reviewed by Bolten, 2003a). The first (Type 1) pattern includes complete development and maturation entirely within the inshore or neritic zone. The only example of a living sea turtle that exhibits the Type 1 pattern is the flatback turtle (*Natator depressus*). The Type 2 pattern is characterized by initial development in the oceanic zone followed by recruitment into the neritic zone. These ontogenetic shifts in habitat utilization are hypothesized to provide maximum growth potential and minimize predation risk (Werner and Gilliam, 1984). The Type 2 pattern is the most common and is characteristic of the life histories of *C. caretta*, *C. mydas*, *E. imbricata*, *L. kempi*, and some populations of *L. olivacea*. In Atlantic *C. caretta*, these shifts appear to be complex and reversible rather than discrete transitions (Witzell 2002; McClellan and Read, 2007). The size and apparent age of recruitment into the neritic zone varies among species. The oceanic stage is the longest for Atlantic *C. caretta*, which return to neritic life after 7 to 11.5 years at a size of 46 to 64 cm (curved carapace length (CCL))(Bjorndal et al., 2003). *Chelonia mydas*, *E. imbricata*, and *L. kempi* are thought to have a shorter ocean life stage, which is estimated to be 3 to 5 years for *C. mydas* (Reich et al., 2007). Size at recruitment into neritic foraging grounds is 20 to 35 cm for *C. mydas* and *E. imbricata*, and 20 to 25 cm for

L. kempi. The third life history pattern or Type 3 pattern is exhibited by *D. coriacea* and East Pacific *L. olivacea*, which undergo development completely within the oceanic zone.

Life history is best characterized for North Atlantic *C. caretta*, which includes Florida's nesting population. Oceanic stage turtles are found in waters near the Azores, Canary Islands, and Madeira Islands where they arrive from natal beaches via the Gulf Stream (Bolten, 2003b). Immature turtles are primarily epipelagic during the oceanic stage, but may become epibenthic or demersal near islands, seamounts, or other formations (Bolten, 2003b). Following recruitment into the neritic zone, *C. caretta* become primarily epibenthic or demersal and utilize a variety of habitats as carnivore generalists. Pelagic feeding aggregations and oceanic migration routes also have been identified for *C. caretta* originating from other rookeries (Musick and Limpus, 1997; Bowen and Karl, 2007). There are far fewer reports of oceanic *C. mydas*, thus less is known about the life history of this species. A key difference between the *C. caretta* and *C. mydas* is that neritic *C. mydas* primarily are herbivorous and feed on seagrasses and macroalgae.

Differences in life history of sea turtle species result in differences in the size classes encountered in different regions, which is reflected in the stranding data. In Florida, peaks in size frequency distribution based on curved carapace length (CCL) of stranded *C. caretta* are between 60 to 80 cm and 90 to 105 cm CCL (Foley et al., 2007). In contrast, peaks for *C. mydas* are 30 to 40 cm and 100 to 105 cm; therefore, most stranded immature *C. mydas* are much smaller than the average immature *C. caretta*. These comparisons do not include stranded post-hatchlings, which are another major size class represented in the stranding data for both species.

Spirorchiid Trematodes

Family diagnosis

The family Spirorchiidae Stunkard, 1921 is comprised of intravascular trematode parasites of freshwater and marine turtles. These parasites primarily are vascular generalists and

various species inhabit arterial, venous, and lymphatic systems (Smith, 1997a). Extravascular migration has been observed in some freshwater forms (Smith, 1997a). The morphology of spirorchiids is diverse and ranges from linguiform to filiform with both distomous and monostomous types. All known members are hermaphroditic. Egg morphology also is highly variable in size and shape. One or two polar filaments may be present and an operculum may be present or absent. Life cycles have only been discovered for freshwater forms, which all utilize gastropod mollusks as a single intermediate host (Smith, 1972; Smith, 1997a). Freshwater spirorchiid cercariae develop through one or more sporocyst generations. Development of rediae has not been reported. Cercariae are brevifurcate and apharyngeate. Freshwater chelonian hosts are infected by penetration through the oral, nasal, conjuctival and cloaca integument and mucous membranes (Smith, 1997a).

Taxonomy and phylogeny

The first spirorchiid was described by Leared in 1862 as a parasite of the "edible turtle" or "common turtle," which may have been *Chelonia mydas* (Smith, 1972). Almost 100 spirorchiid species within 19 genera have been described over the last nearly 150 years (Smith, 1997a; Platt, 2002). Reported host chelonian species represent all major families except Testudinidae (Platt, 1992). Records are biased toward sea turtles and North American freshwater turtles. Many new spirorchiids undoubtedly will be discovered as it is estimated that only around 15% of chelonian species have been examined for these parasites (Snyder, 2004).

Spirorchiidae is within the superfamily Schistosomatoidea (Trematoda: Digenea: Strigeadida), which also includes Sanguinicolidae and Schistosomatidae. Sanguinicolidae are blood flukes of fish and Schistosomatidae includes blood flukes of mammals, birds, and crocodilians. Recent phylogenetic studies of Schistosomatidae and Spirorchiidae based on small and large subunit ribosomal DNA support that marine spirorchiids form a clade sister to the

schistosomatids (Snyder, 2004). Spirorchiids of freshwater turtles are basal to marine spirorchiids, suggesting that marine forms are derived from a freshwater ancestor (Snyder, 2004). In addition, this study also demonstrated that Spirorchiidae is in fact paraphyletic, and thus taxonomically invalid (Snyder, 2004). This finding likely will result in revision of higher classification and thus additional complexity in the progression of spirorchiid taxonomy.

The taxonomy of Spirorchiidae is confounded by divergent morphology, a limited specimen database, poorly preserved/partial specimens, incompletely described species, and inaccessible specimens (Platt, 2002). The literature is widely dispersed and replete with disputes over nomenclature and synonymy. There have been revisions and redescriptions of multiple taxa (Dailey et al., 1993; Platt, 1993; Platt and Blair, 1998; Platt, 2002). Even the spelling of the family name has been revisited, and appears in the literature as both Spirorchidae, as proposed by Stunkard (1921), and Spirorchiidae, which was proposed by MacCallum later the same year. MacCallum's spelling, although not etymologically correct, is preferred by the International Commission on Zoological Nomenclature (ICZN) (although Stunkhard is credited with the discovery) because many other digeneans with "-orchis" family names have an "ii" ending (Platt, 2002). An official ruling has not been rendered by the ICZN. Subdivision of Spirorchiidae into subfamilies has been proposed (Azimov,1970; Yamaguti, 1971; Smith, 1972), but these schemes are unsupported by phylogenetic analysis (Snyder, 2004).

Marine spirorchiids

Ten genera of marine spirorhids are recognized in a recent compilation of keys for the family Spirorchiidae (Platt, 2002). However, four of these genera, *Cheloneotrema* (Simha & Chattopadhyaya, 1976), *Neocaballerotrema* (Simha, 1977), *Satyanarayanotrema* (Simha & Chattopadhyaya, 1980), and *Shobanatrema* (Simha & Chattopadhyaya, 1980), have only been described from the Indian subcontinent and have not been available for subsequent review (Platt,

2002). Platt noted that the descriptions of these parasites are insufficient to distinguish them from other previously described genera. In fact, he proposed that a fifth Indian genus, *Squaroacetabulum* (Simha & Chattopadhyaya, 1970), is a junior synonym of *Amphiorchis* (Platt 2002). Table 1-1 includes reports of spirorchiids of sea turtles in the peer-reviewed literature. Excluded are abstracts and identifications beyond the generic level that were based solely on egg morphology, except where data were reasonably substantiated and contributed to either geographical or host range. Many of these references were previously compiled by Smith (1997b). Modifications include recognized synonymies, reports since 1996, and additional data on parasite microhabitat, associated pathology, and prevalence data. Many of these taxa probably should be further studied and re-evaluated for validity, as has been done for *Hapalotrema* (Cribb and Gordon, 1998; Platt and Blair, 1998) and *Carettacola* (Dailey et al., 1991).

Reported sea turtle host species for spirorchiids are limited to *C. mydas*, *E. imbricata*, and *C. caretta* (Smith, 1997b). There are no published descriptions of spirorchiids of *Lepidochelys*, *Natator*, or *Dermochelys*. Approximately fourteen spirorchiid species within eight genera have been described from *C. mydas*, eight species within three genera from *E. imbricata*, and six species within five genera from *C. caretta* (Table 1-2). Smith (1997) stated as many as 22 species within nine genera had been reported for *C. mydas*. The lower number given here reflects reported synonymies, exclusion of those reports without specific identification, and two cases where definitive host identification was ambiguous or unsubstantiated (Leared, 1862; Mehrotra, 1973; Gupta and Mehrotra, 1981).

Several marine spirorchiid species have been reported to parasitize multiple sea turtle species. As noted by Smith (Smith, 1972; Smith, 1997a), there is no evidence of “strict

‘phylogenetic host specificity’ at either the generic or species levels. Many freshwater spirorchiid species are known to parasitize multiple chelonian host species, including chelonian species of different genera (Platt, 1993; Smith, 1997a). In contrast to this opinion, one study of digenean trematodes in Caribbean *C. mydas* categorized some spirorchiids as “generalists,” indicating parasitism of multiple host species, and others as “specialists,” indicating parasitism of a single host species (Santoro et al., 2006). This assumption of specificity may be premature given the limited information or lack of data for sea turtles in many regions. Perceived host specificity may reflect selective exposure to intermediate hosts, as dictated by habitat utilization, rather than true specificity.

Reports of several spirorchiid species support a worldwide distribution for some taxa. *Hapalotrema pambanensis*, *Hapalotrema mistroides*, *Hapalotrema postorchis*, *Hapalotrema synorchis*, *Learedius learedi*, *Montecellius indicum*, and *Neospirorchis schistosomatoides* have all been reported from distant regions (see Table 1-1). The spirorchiid literature is heavily biased toward *C. mydas*, both in the number of reports and the numbers of individual turtles examined. In a 1972 review, Smith stated that:

> “To undertake [a detailed description of world distribution] using known records would be premature and probably reflect only the past and present world distribution of marine and freshwater research institutes, the attractiveness and/or accessibility of particular localities to research workers, and a lack of interest in the group.”

This statement is still valid as sea turtles inhabiting many geographical regions have yet to be studied or have received only limited study. The only marine spirorchiid species for which a restricted geographical distribution has been proposed is *Carettacola hawaiiensis*, which has only been identified from *C. mydas* in Hawaii. Work et al. (2005) propose that *C. hawaiiensis* is an endemic parasite, whereas *Hapalotrema* species and *Learedius* were introduced. This hypothesis is based on limited reports of the parasite, small parasite numbers in the turtle host,

and minimal evident pathology. The latter were interpreted as evidence of a longer, more evolved host-parasite relationship as compared to other spirorchiids. However, spirorchiid data from other areas of the Pacific, other than Australia, are very limited and phylogenetic studies to support this hypothesis are lacking.

Prevalence, microhabitat, and host pathology

Most of the published reports of marine spirorchiids fall into the categories of primary parasitological descriptions/studies and studies of associated host pathology. The former often lacks mention of associated pathology and the latter specific identification of parasites. Thus, the interpretation of significance of parasitism, in terms of health implications in the turtle host, frequently is disconnected from specific identification of the parasites. Also, most reports are limited to small numbers of turtles, thus prevalence cannot be inferred.

Of those publications cited in Table 1-1, only eleven (excluding those cited by Smith that are unavailable) have included data from ten or more wild sea turtles (Looss, 1899; Looss, 1902; Fischthal and Acholonu, 1976; Glazebrook et al., 1981; Wolke et al., 1982; Rand and Wiles, 1985; Glazebrook et al., 1989; Glazebrook and Campbell, 1990; Dailey et al., 1991; Dailey et al., 1992; Gordon, et al., 1998; Work et al., 2005; Jacobson et al., 2006; Santoro et al., 2006). Comparison of prevalence among most existing studies, as attempted by Glazebrook et al. (1990), is unsupported due to differences in methods of detection, inadequate descriptions of methodology, and examination of different age/size classes and species of turtles. For example, Wolke et al. (1982) reported that 14 of 43 accessions had spirorchiids; however, only sixteen complete carcasses were examined. Several studies report a combined prevalence of multiple spirorchiid genera and species (Glazebrook and Campbell. 1990; Gordon et al., 1998; Work et al., 2005), or a combined prevalence that includes multiple sea turtle species (Glazebrook et al., 1981; Glazebrook et al., 1989).

Detection of spirorchiids and associated lesions requires a targeted approach that includes meticulous examination of vascular compartments, dissection of the cardiovascular system, examination of all tissues (both grossly and histologically), and fecal examination. In those studies that apply targeted methodology, spirorchiid prevalence in many study populations is greater than 95% (Dailey et al., 1992; Gordon et al., 1998; Work et al., 2005; Santoro et al., 2006). A study of digeneans in nesting female *C. mydas* in Tortuguero National Park, Costa Rica, by Santoro et al. (2006) is especially notable because this study population was comprised of nesting females killed by jaguar, and thus is most representative of a large group of otherwise healthy turtles. Many of the other studies primarily included dead or moribund stranded turtles or turtles with other health problems, such as fibropapillomatosis. Work et al. (2005) used enzymatic digestion of the spleen, followed by the use of Flukefinders (a differential filtration technique) to recover spirorchiid ova as a means, although unvalidated, of estimating tissue ova numbers in *C. mydas*. This method appears to be quite effective for detection given that all 99 *C. mydas* included in this study were found to be infected.

Parasite microhabitat selection or host-organ/tissue fidelity has obvious implications on associated effects in the host and biology of the parasite, e.g. successful propagation. In the marine and freshwater spirorchiid literature, there are examples suggesting varying degrees of host-organ fidelity, as well as reports that some species do not exhibit specific tropism. This aspect of spirorchiid biology is somewhat obscured by vague descriptions of parasite locations within the host and lack of information on the parasite maturity in some reports.

By far, the heart and major arteries, including the pulmonary arteries and regions of the aorta, are the most commonly reported sites from which adult marine spirorchiids have been recovered (Table 1-1). It must be considered that there is at least some bias in the literature as

these sites are easily assessed, and other organs and tissues, such as brain and smaller vessels, are more technically difficult to extract and thoroughly examine, especially in large turtles. The heart and major arteries are consistently mentioned in reports of *Hapalotrema* and *Learedius* species. *Montecellius indicum* also is described from the heart in the two published studies. Multiple authors describe *Carettacola* species from hepatic and mesenteric vessels. Both *Neospirorchis pricei* and *N. schistosomatoides* have been described from the heart, *and N. schistosomatoides* also has been reported in meningeal vessels. Other genera and species are limited to reports in single animals, thus consensus observations on possible tropisms are not possible. Furthermore, in many other reports given in Table 1-1, the location of parasites is very vague, such as "visceral blood vessels," "blood vessel," or "circulatory system," or location is not identified.

There are reports of both host-organ specificity and lack thereof in the freshwater spirorchiid literature. Wall (1941) reported a specific tropism of *S. elephantis* for gastroenteric arterioles. Another spirorchiid, *Vasotrema robustum*, is described as undergoing early development in hepatic vessels followed by migration to the heart and major arteries (Wall, 1951). In contrast, multiple authors report a lack of host-organ specificity, as well as migration of adults outside of vessels, in species of the genus *Spirorchis* (Smith, 1972; Smith, 1997a). In two different studies of experimentally infected *Chrysemys picta picta*, *Spirorchis scripta* and *S. parvus* were collected from a variety of anatomic locations, including non-vascular sites (Holliman and Fisher, 1968; Holliman et al., 1971). In both studies, it was stated that adult and immature parasites were collected; however, the maturity of the parasites from the various locations was not clearly indicated. In addition, Holliman et al. (1971) argue that their findings confirm a lack of host-organ specificity for adult *S. parvus*, yet they report that 217 of 247

recovered parasites were removed from the central nervous system. This finding arguably suggests a tropism for the central nervous system or its associated vasculature. In addition, some of the turtles in this study were infected with both *S. parvus* and *S. scripta*, and it appears that this dually infected group is included in two different publications (Holliman and Fisher, 1968; Holliman et al., 1971). Although the recovered spirorchiids were specifically identified, it is unclear if the locations from which they were recovered were combined because the reported numbers are inconsistent. It is reported that 217 *S. scripta* were recovered from the singly infected group and 23 from the dually infected group (240 total *S. scripta*); however, anatomic locations are listed for 230 specimens (Holliman and Fisher, 1968). In considering this issue of host-organ fidelity in *Spirorchis* sp., Platt (1993) notes finding *S. parvus* in both the "cranial and mesenteric circulation" of naturally infected *C. picta marginata*. Lastly, lack of host-organ specificity also is reported for *S. elegans* in *C. picta picta*, although, again, maturity of parasites from various sites was unclear (Goodchild and Kirk, 1960).

The host effects and pathological lesions associated with spirorchiidiasis have been studied in both freshwater and marine turtles. The reported effects of spirorchiid infection fall into the categories of vascular and tissue injury and inflammation associated with adult parasites and eggs and more general, nonspecific descriptions of weight loss, lethargy, and chronic debilitation. The hypothesized association between fibropapillomatosis and spirorchiids will be discussed separately. Studies of the pathologic effects of marine spirorchiids primarily have examined *C. mydas* (Looss, 1902; Glazebrook et al., 1981; Rand and Wiles, 1985; Glazebrook, et al., 1989; Glazebrook and Campbell, 1990; Graczyk et al., 1995; Gordon et al., 1998; Raidal, et al., 1998; Cordero-Tapia et al., 2004; Work et al., 2005), fewer *C. caretta* (Looss, 1902; Wolke et al., 1982; Glazebrook and Campbell, 1990; Jacobson et al., 2006), and a small number

of *E. imbricata* (Glazebrook et al., 1989; Glazebrook and Campbell, 1990). In most of these studies, examined turtles were parasitized by multiple spirorchiid species and little or no effort is made to associate lesions with specific species.

One of the earliest references to pathological lesions associated with spirorchiids was by Looss (1902), who noted changes in the vascular intima and endothelium associated with attached *Hapalotrema* in his studies of *C. mydas* and *C. caretta* in Egypt. Looss also noted a lack of correlation between the numbers of spirorchiid eggs observed and the number of adult flukes present.

Wolke et al. (1982) reported spirorchiidiasis and associated lesions in 14 of 43 accessions, which included random tissues and 16 whole carcasses of *C. caretta* that stranded on the Atlantic coast of the United States. Among these accessions, the authors describe an unspecified number of chronically debilitated, severely emaciated turtles with heavy epibiota coverage and associated these cases with large numbers of spirorchiid ova in various tissues. It is stated that 90% of lesions were "focal granulomata" and two morphological forms of granulomas were described, one characterized by central necrosis and a second characterized by giant cells formation without necrosis. The authors also note cases of bacterial enteritis secondary to spirorchiid egg deposition, although the type of spirorchiid is not indicated. Spirorchiid egg morphologies representing the three main types are noted, but adults were not collected. Lastly, the finding of iron accumulation in the liver, spleen, and kidneys was interpreted as evidence of hemolytic anemia, which is speculated to have contributed to the pathogenicity of spirorchiid infection.

A report of pathological changes associated with spirorchiidiasis in a single moribund *C. mydas* moribund from Australia was described by Glazebrook et al. (1981). The affected turtle

was chronically ill and had large numbers of spirorchiids and mycotic pneumonia. Many (167) *Hapalotrema* sp. were recovered in the cardiac ventricle and base of the right aortic arch and brachiocephalic artery, where there was localized endocarditis and endarteritis and thrombus formation. Spirorchiids also were noted in the brain and other organs, and were presumed to be the same species, but were not identified. These authors describe a very similar case or the same turtle in a second study eight years later and present a photomicrograph of an egg mass and adult spirorchiid within the meninges that is identifiable as a *Neospirorchis* sp. (Glazebrook et al., 1989). The parasite is misidentified as *Hapalotrema* sp. in both papers. Large numbers of spirorchiid eggs were observed in multiple organs and were associated with granuloma formation. In the 1989 study, the authors reportedly examined 109 farmed turtles, 20 oceanarium turtles, and 39 wild turtles. Most presumably were *C. mydas* and an unspecified number of *E. imbricata* also were examined. The wild turtles had both the highest prevalence (77.2% (17/22)) and heaviest infections. Prevalence in farmed and oceanarium turtles was 4.8% (5/104) and 33.3% (5/15), respectively. Although the oceanarium turtles are identified as "oceanarium-reared," it appears from a later publication that some of these turtles were captured as subadults or adults and may have been infected prior to captivity (Glazebrook and Campbell, 1990). Clinical signs were observed in only 26% of turtles with spirorchiidiasis and were described as nonspecific, including emaciation. Many spirorchiids are recovered from the heart and major vessels (primarily the right aortic arch and brachiocephalic artery), and adults are noted in other organs, including the central nervous system (likely *Neospirorchis* sp.), spleen, lungs, and skeletal muscle. The spirorchiids collected, *Hapalotrema* sp. from *C. mydas* and *E. imbricata* and *Learedius*, were identified to genus only. Gross vascular lesions described as thickening and hardening of the arterial walls were observed in 5 of 17 infected wild turtles and three had

associated thrombi. Two animals also had chronic pneumonia, although it is unclear if this lesion was associated with embolization of eggs. Proliferative endocarditis and endarteritis are described as is varying degrees of granulomatous inflammation in multiple organs associated with egg embolization. The authors also described congestion and edema, which they associated with embolized eggs. Much of these data appear to have been published again by Glazebrook and Campbell (1990) in a subsequent survey of diseases of oceanarium-reared and wild turtles.

Rand and Wiles (1985) examined eleven moribund or dead *C. mydas* recovered in Bermuda and report identification of *L. learedi* and *N. schistosomatoides* in the group. Description of lesions is limited to granulomatous inflammation associated with spirorchiid eggs in tissues. Other findings are nonspecific and include findings typical of chronically ill turtles, such as atrophy of fat and soft tissue edema. Significance of spirorchiidiasis in these cases was not specifically evaluated.

A brief description of lesions associated with spirorchiid infection in a single *C. mydas* from Hawaii was given by Graczyk et al. (1995) in a study of spirorchiid exposure using an enzyme-linked immunosorbent assay (ELISA). Generalized lymphoplasmacytic endarteritis, thrombosis, and granuloma formation in multiple organs associated with spirorchiid eggs is described in an emaciated turtle with fibropapillomatosis. Parasites from this turtle included *H. dorsopora* (syn. *pambanensis*), *L. learedi*, and *C. hawaiiensis*, which were used as source of antigen for the ELISA.

One of the largest and most in-depth studies on the impact of spirorchiids in sea turtles was an examination of associated pathological lesions in stranded *C. mydas* in Queensland, Australia (Gordon et al., 1998). This was the first study to clearly address some of the more important aspects of spirorchiidiasis, such as distinguishing incidental (to the cause of death)

infections from significant and fatal pathological lesions. Other studies have mentioned incidental infections, but often failed to provide actual numbers or apparently did not have complete necropsy data on which to base confident interpretation. In the study by Gordon et al (1998), the criteria for categorizing significance, however, were not specifically stated. Spirorchiid infection was observed in 97.9% (94/96) of examined green turtles, with 30.2% (29/96) of the turtles having one of multiple severe problems due to spirorchiidiasis, and 10.4% (10/96) dying directly from spirorchiid infection. It appears that most of the turtles examined were chronically ill, as indicated by emaciation, and all had ascites and “anasarca,” which the authors were unable to correlate with decreased plasma protein. Pathological lesions included mural endocarditis (33/96), localized near the atrioventricular junctions, and endarteritis. Remarkably, arterial thrombosis is recorded in 81 turtles, and both mural thrombi and extensive occlusive thrombi are described. Aneurysm formation is reported as a common finding and the authors illustrate an unusual process of exteriorization of thrombi from the vessel lumen. Septic thrombosis was a confounding problem in six turtles. The host response is as described by previous authors and includes granuloma formation accompanied by granulocytes and lymphocytes in most organs and tissues. Concentrations of egg granulomas that were considered significant were observed in the lungs of six turtles, and in one case a more acute, i.e. granulocytic response is described. Histologically, the cardiac and vascular lesions were characterized by papillary proliferation and subintimal stromal proliferation, in addition to the inflammatory infiltrate and pigmented and mineralized debris. Similar changes also were observed in splenic, pulmonary, and pancreatic vessels. The authors could not confirm correlation of spirorchiids with circulatory disturbances, such as congestion, edema, or hemorrhage. Spirorchiid species are identified from a subset (n=8) of affected turtles, and

include *H. pambanensis* (syn. *mehrai*), *H. postorchis*, and *N. schistosomatoides*. Most spirorchiids were recovered from the left aorta, and fewer from the right aorta and heart. In addition, the authors refer to flukes as "macroscopic" and "microscopic," with the latter referring to flukes that were only identified in tissue section. It appears that many of these likely were *Neospirorchis* species, based on the authors' description of a filiform body type. These parasites were observed in a variety of sections, including brain (53/72), gastrointestinal tract (15/69), lungs (7/70), liver, thyroid gland, adrenal gland, and periarterial connective tissue. Associated meningeal lesions are described as considerably milder than in other organs, and were only considered significant in one turtle that had severe granuloma formation and hemorrhage. "Serpiginous" lesions are described in the blood vessels of the meninges and enteric submucosa that may have been *Neospirorchis* egg masses based on the description, but this is unclear. Lastly, the authors note a lack of correlation between the severity of lesions and the numbers of flukes recovered and state that this disparity in observations, concurrent disease processes, chronic stage of disease in stranded turtles, and lack of knowledge regarding spirorchiid life cycles are major obstacles in characterizing and interpreting health implications of spirorchiidiasis.

Raidal et al. (1998) report four cases of spirorchiidiasis associated with Gram-negative bacterial infections in immature green turtles that stranded in western Australia. Parasitism by multiple species of spirorchiids is described and is associated with granulomatous vasculitis and, in two cases, thrombosis. Lesions supporting concurrent bacterial infection only are presented for three of the four cases. Recovered spirorchiid genera included *Amphiorchis*, *Hapalotrema*, and *Carettacola*, which were not identified to species. In addition, a photo of the brain from one

infected turtle demonstrates a large number of spirorchiid eggs within the meninges, which likely was a *Neospirorchis* sp. This finding was observed in two turtles.

Two reports of spirorchiidiasis in black turtles (*C. mydas agassizii*) describe *L. learedi* in turtles killed as by-catch in Magdalena Bay, Mexico (Cordero-Tapia et al., 2004; Inohuye-Rivera et al., 2004). As in previous studies, adult parasites were removed from the heart (three of four examined hearts in this report) and granulomas associated with spirorchiid eggs were found in every tissue examined. An interesting linear arrangement *of L. learedi* eggs in the pancreas is described and interpreted this as possible migration towards the enteric lumen (Cordero-Tapia et al., 2004). The prevalence of infection in these studeis is unclear.

Work et al. (2005) used splenic spirorchiid egg numbers as an estimate of intensity of spirorchiid infection and compared these data to various parameters, including turtle size, body condition, fibropapilloma diagnosis, and spirorchiid serological status in a study of Hawaiian *C. mydas*. The use of splenic egg numbers as a surrogate for evaluation of the entire animal was not validated in this study. Spirorchiid eggs were detected in all of the examined turtles. Splenic egg burden was found to significantly decrease with increasing straight carapace length (SCL) and increasing body condition index (BCI). These results indicated that factors related to host age influence spirorchiidiasis, which also has been suggested in studies of freshwater turtles (Holliman, 1971). A trend in turtles from different regions also was identified.

Spirorchiids and serological studies

Serological assays for the detection of exposure to spirorchiid trematodes have been used in three different studies (Graczyk et al., 1995; Herbst et al., 1998; Work et al., 2005). Graczyk et al. (1995) developed an indirect ELISA using combined preparations of crude surface glycocalyx antigen from *L. learedi*, *H. dorsopora* (syn. *pambanensis*), and *C. hawaiiensis*. In this study, plasma from 58 wild turtles (5 localities, but 41 turtles from one locality) was tested

and seroprevalence was 100% from all localities, except Ahu-O-Laka, Kaneohe Bay, Oahu. Herbst et al. (1998) used total crude antigen from *L. learedi* and an unidentified *Hapalotrema* sp. to develop an indirect ELISA for studying serological exposure of *C. mydas* with and without FP to herpesvirus and spirorchiids. Examined turtles included a group with experimentally produced FP, turtles from Indian River Lagoon with a high prevalence of FP, and turtles from reef habitat where FP is not observed. No significant association was found between spirorchiid seroprevalence and habitat. Interestingly, seropositive animals included the smallest turtle tested (SCL = 26.8 cm). Cross-reactivity was noted between *Learedius* and *Hapalotrema*, as well as *Neospirorchis* and gastrointestinal digeneans, but was more specific when a higher cutoff value was applied. Using a higher cutoff limit, a lower seroprevalence was found in FP turtles as compared to FP-free turtles, which did not support an associated between spirorchiids and FP. Proposed explanations for these findings included immunosuppression or immunomodulation in FP turtles, and that the lagoon may not support active spirorchiid infection. As stated by the authors, all of these possibilities require significant additional studies. In a recent study, Work et al. (2005) developed ELISA's to detect antibodies against adult and egg crude antigen using combined preparations from *Learedius* sp., *Hapalotrema* sp., and *Carettacola hawaiiensis*. Animals in this study included wild hatchling, immature, and adult turtles from different localities, as well as captive-bred and reared turtles, some of which were exposed to coastal habitat. Captive turtles in filtered seawater had the lowest titers, followed by hatchlings, which were thought to have maternal antibody. Equivalent exposure was noted in wild immature and adult turtles. Oceanic stages were not examined is any of these studies.

Pathology associated with freshwater spirorchiids

Many of the descriptions of host pathology associated with freshwater spirorchiids are reported in the context of experimental infections during life history studies. Irritation of the

head region manifested by blinking, gaping, and “vigorous” clawing is described with exposure to infectious cercariae (Holliman and Fisher, 1968; Holliman et al., 1971). Other clinical signs generally are nonspecific and some reported associated findings, such as osteopenia, are not confidently attributable to spirorchiidiasis (Holliman and Fisher, 1968). Reported clinical signs include listlessness, stupor, and edema of the head and thoracic limbs. There also are references to higher mortality in experimentally infected turtles (Holliman and Fisher, 1968; Holliman et al., 1971). Much of the described associated host injury and inflammation is associated with eggs within various tissues (Wall, 1941; Goodchild et al., 1967; Johnson et al., 1998). Holliman (1971) made the interesting observation that hyperinfected turtles may contribute to the parasite life cycle by releasing eggs, including those trapped within tissues without apparent alternative means for reaching the external environment. In addition, there are two reports that include neurological symptoms in freshwater turtles associated with spirorchiids. Holliman et al. (1971) report one case of hemiplegia and circling in a *Chrysemys picta picta* due to a necrotic brain lesion associated with *S. parvus*. Similar neurological signs also are reported by Johnson et al. (1998); however, there were no correlative pathological findings presented to explain the clinical signs. Other associated pathology findings in freshwater turtles include proliferative endarteritis in captive *Trachemys scripta* elegans and *Chrysemys picta* (Johnson et al., 1998).

Fibropapillomatosis and spirorchiids

A review of the marine spirorchiid literature would be incomplete without mention of fibropapillomatosis. Considerable effort has been expended in investigating a possible association between spirorchiids and this disease. Spirorchiid eggs were first reported within FP tumors by Smith and Coates (1939). These authors ultimately concluded that the parasite ova probably were not the “immediate cause of the disease” based on further examination of two additional turtles with FP that did not have evidence of spirorchiid infection. An interest in FP

re-emerged in the 1980's as a high prevalence of tumors was observed in *C. mydas* in Florida and Hawaii (Balazs, 1986; Erhart, 1991). Spirorchiids were again revisited as causative factor in the pathogenesis of FP based on presence of eggs in some tumors and the precedence for fibrogenic host response, papillomatous lesions, and malignancy associated with mammalian schistosomes (Smith and Coates, 1939; Harshbarger, 1984). Subsequent studies, however, did not support a causative association between spirorchiids and FP. There were two unsuccessful attempts to induce FP by injecting spirorchiid material (Herbst, 1994; Dailey and Morris, 1995). In addition, two independent serological studies, one in Florida and one in Hawaii, did not indicate any association between exposure to spirorchiids and FP (Graczyk et al., 1995; Herbst et al., 1998). Additional studies offered compelling evidence that implicated a novel herpesvirus as the primary agent of concern (Jacobson et al., 1991; Herbst and Klein, 1995; Herbst et al., 1998; Quackenbush et al., 1998). Studies of FP have turned to further characterization of the associated herpesvirus, virus variants, and other possible contributing factors, such as toxins and pollutants. Most, if not all investigators have largely disregarded any significant role of spirorchiids in the pathogenesis of FP.

Life cycles

As previously stated, the life cycles of all marine spirorchiids are unknown, thus important aspects of epidemiology, disease ecology, and other critical features of spirorchiidiasis, e.g. time course of parasitism, have yet to be studied. Inability to discover the life cycles of marine spirorchiids is a significant obstacle to better understanding of these parasites and their implications on sea turtle health (Gordon et al., 1998). The life cycles of several freshwater spirorchiids, however, have been identified, and may offer some insight into intermediate hosts of marine forms. In addition, other information relevant to marine life cycles includes evidence of disparity in spirorchiid prevalence among different life stages of sea turtles,

as well as documented infections in captive-hatched or captive-bred turtles at coastal facilities. These data can be used to develop hypotheses regarding the general habitats in which infections are acquired and studies of potential intermediate hosts.

The life cycles have been characterized and experimentally reproduced for as many as seven species of freshwater spirorchiids (depending on resolution of the species inquirenda status of some *Spirorchis* species) (Smith, 1997a). There is a bias for temperate North American species in these studies. All spirorchiid life cycles include a single gastropod mollusk intermediate host. The only examples of trematodes within the superfamily Schistosomatoidea that utilize non-gastropod intermediate hosts are some species of sanguinicolids, which parasitize lamellibranch mollusks (bivalves) and polychaete annelids (Smith, 1997a). In fact, sanguinicolids are only digeneans known to utilize non-gastropods as primary intermediate hosts. Nonetheless, given the basal phylogenetic position of freshwater spirorchiids relative to marine forms, and the consistent utilization of gastropod mollusks by schistosomes, it reasons that marine spirorchiids most likely also utilize gastropod mollusks as intermediate hosts.

Gastropods that serve as intermediate hosts of freshwater spirorchiids include species within the genera *Helisoma*, *Physa*, *Menetus*, *Ferrissia*, and *Indoplanorbis* (Smith, 1997). Prevalence of spirorchiid infection in snail populations is low in many studies with evidence of seasonal variation. Holliman (1971) examined 3,502 *Helisoma anceps* and found that only 1.28% were infected with *S. scripta* and 0.11% with *S. parvus*. Holiman hypothesized that “successful completion of the life cycles was more dependent on high cercarial productivity of the individual snail rather than on the number of snails infected.” Turner and Corkum (1977) conducted a 16-month survey of digenean infections in three species of ancylid snail in Louisiana. *Spirorchis scripta* only was found in *Ferrissia fragilis*, with a peak prevalence in

July (study period was April through July) of only 1.3%. A remarkable seasonal prevalence and the highest prevalence recorded thus far for any spirorchiid in its intermediate host was reported in snails inhabiting a reservoir in Kentucky (Rosen et al., 1994). Infection of the snail *H. anceps* by *S. scripta* had peak prevalence of 18.2% in May to June that decreased into mid-summer and remained undetectable from November through April. Several freshwater spirorchiid life cycle studies have indicated that adult snails are less susceptible or resistant to infection (Wall, 1941; Holliman and Fisher, 1968; Holliman, 1971; Holliman et al., 1971; Rosen et al., 1994). Rosen et al. (1994) documented a higher prevalence of infection in smaller snails, suggesting a rapid, parasite-associated mortality in the smaller cohort. In contrast, Goodchild and Fried (1963) did not observe any age-related susceptibility to spirorchiid infection in experimentally infected *Menetus dilatatus buchanensis*.

Spirorchiid cercariae develop through one or more generations of sporocyst and, as with many aspects of spirorchiid development, the rapidity of this process is temperature dependent (Wall, 1951; Goodchild and Kirk, 1960; Holliman and Fisher, 1968; Smith, 1972). Cercarial output varies with some reports of 100 or more in a single day (Pieper, 1953; Holliman, 1971). Infected snails can be quite long-lived with survival records as long as two to several months (Pieper, 1953; Goodchild and Fried, 1963). As with other blood flukes, emerged cercariae infect the turtle host by penetration. Experimentally exposed turtles are observed to exhibit irritation around the eyelids and proposed sites of penetration include the eyelids, conjunctiva, nares, oral cavity and cloaca (Wall, 1941; Wall, 1951; Goodchild and Kirk, 1960; Holliman and Fisher, 1968; Holliman et al., 1971).

Experimental reproduction of the life cycles of freshwater spirorchiids has provided useful information on infection in turtle hosts. The longevity of spirorchiids in turtle hosts is

poorly defined. Holliman (1971) reported finding an adult spirorchiid in the mesentery 293 days after exposure to *S. scripta* cercariae. The prepatent periods reported by different studies are widely variable and range from six weeks to as long as a year (Table 1-1). Possible explanations of this disparity include differences in parasite and host species or host interaction, temperature, and detection methods. Parasite maturation and oviposition are reported to be temperature dependent; however, this observation is based on a single study with a small sample size and exposure to different numbers of cercariae, thus additional work is necessary (Goodchild et al., 1967). Holliman (1971) also noted shorter maturation times, as well as larger numbers of parasites, in younger turtles. In a study of *S. parvus* in *C. picta*, investigators noted that eggs were never found in the feces, even from hosts infected as long as 332 days (Holliman et al., 1971). It was further noted that egg numbers were low in the enteric wall of some hosts, which may have limited detection in the feces. After eggs are expelled or extracted from the turtle, hatching occurs in approximately 4 to 8 days and is temperature dependent.

There are two unsuccessful attempts to discover the life cycles of marine spirorchiids in the literature. Greiner et al. (1980) examined several gastropod species found at the Cayman Turtle Farm, Limited (CTFL) where resident *C. mydas* are infected with *L. learedi*. A second study is briefly mentioned by Dailey in a survey of digenean trematodes of Hawaiian *C. mydas* (Dailey et al., 1992).

Although an intermediate host has yet to be identified, there is evidence that at least some species infect turtles in the neritic or inshore zone. *Learedius learedi* infects captive-bred *C. mydas* at the CTFL (Greiner et al., 1980). Gastropods and other organisms are not commonly found in the rearing tanks at the CTFL, which suggests that infectious cercariae are brought into the facility via seawater and that the intermediate host lives in the adjoining marine habitat

(Greiner et al., 1980). There are two additional examples indicating infection in the neritic zone. Captive-hatched *C. mydas* housed at a rehabilition facility in Marathon Key, Florida (The Turtle Hospital) were found to become infected by species of *Neospirorchis* (L. Herbst, personal communication). Lastly, Work et al. (2005) detected antibodies against spirorchiids in captive turtles that were transferred from captivity into coastal seawater ponds.

It also is valuable to compare the prevalence of spirorchiids in different life stages of sea turtles, as this may indicate habitats that support spirorchiid transmission. These data are patchy at best because there are knowledge gaps for some life stages and investigators often neglect to include detailed measurements of the turtle host in spirorchiid studies. There are, however, several relevant references. Oros et al. (2005) did not observe any spirorchiids in 88 immature *C. caretta* recovered from the Canary islands, Spain. This study population was primarily comprised of oceanic stage turtles based on carapace length, which suggests that *C. caretta* are infected following arrival in the neritic habitat. Very limited information is available on oceanic stage *C. mydas* and *E. imbricata*. Looss (1902) noted that *Hapalotrema* sp. already was present in *C. mydas* at 20 to 30 cm (carapace length), which is the approximate size oceanic *C. mydas* recruit into the neritic zone (Musik and Limpus, 1997). Glazebrook et al. (1989) noted that "disease" was not present in hatchling (possibly referring to post-hatchling) or juvenile *C. mydas* and *E. imbricata*, and that all infected turtles from one examined locality were greater than 28.5 cm (curved carapace length (CCL)). These authors do not indicate whether or not smaller turtles from other localities were infected or the numbers of examined turtles within different size classes. Work and Balazs (2002) did not find spirorchiids in two *C. mydas* captured as bycatch in the North Pacific. Both of these turtles were over 55 cm in straight carapace length. No serological studies have included oceanic stage turtles.

A few parasitological surveys have not found spirorchiids in sea turtles despite methods that presumably would have detected infection and, in some cases, relatively large sample sizes. Spirorchiids are conspicuously absent from two parasitological surveys of *C. caretta* (33 and 14 individuals) and *C. mydas* (seven individuals) in the Mediterranean region (Sey, 1977; Manfredi et al., 1998). Neither study gives the sizes of the examined turtles, which may have been oceanic stage animals. Lastly, no spirorchiids have been observed in *D. coriacea* or *L. olivacea*, both of which are believed to exhibit a completely oceanic life history pattern (Manfredi et al., 1996; Work and Balazs, 2002; Oros et al., 2005).

Molecular studies of digeneans and applications to spirorchiid research

Parasite identification and taxonomy based solely on morphology has a number of limitations, many of which are highly relevant to the study of spirorchiid trematodes. These problems can be addressed by judicious use of molecular studies. Morphology-based classification is limited due to the small size of some adult parasites, inability to specifically identify larval or developing life stages, subjective measures of descriptive characters, phenotypic plasticity, morphological similarities between closely related species, and the lag time between speciation events and evolution of observable morphological differences (Nolan and Cribb, 2005). In addition, partial or poor quality specimens, inadequate descriptions, and sparse specimen database have been implicated in the many taxonomic uncertainties of spirorchiids (Platt, 2002). A primary focus of this dissertational research was the use of molecular approaches to identify and detect spirorchiids, and assess for diversity. The following literature review will concentrate on application of ribosomal and mitochondrial genetic data for these purposes.

Internal transcribed spacer regions of the ribosomal gene. Ribosomal DNA is present in the genome as a series of repeats and includes two internal transcribed spacer regions (ITS 1

& 2) separated by the relatively short 5.8s segment. The secondary structure of ribsomsomal sequence must be maintained to sustain function, thus the region stays relatively conserved. The ribosomal repeats evolve in a concerted manner via unbiased gene conversion and unequal crossing over, which is thought to rapidly homogenize and fix new variants within reproductive units. Thus, individuals are believed to be representative of the interbreeding group (Morgan and Blair, 1995; Nolan and Cribb, 2005). However, Vilas et al. (2005) point out that concerted evolution may be slower than assumed in platyhelminths, and resultant hybridization, incomplete lineage sorting, and retention of ancestral polymorphism could confound studies. Nonetheless, the ITS are commonly used markers for species studies and studies of cryptic diversity, and are useful for these purposes in digeneans when appropriately applied (Nolan and Cribb, 2005).

Some studies report greater variability in the ITS1 as compared to the ITS2; however, this finding appears to be inconsistent (Vilas et al., 2005). The ITS1 includes a variable 5' region and more conservative 3' region (von der Schulenberg et al., 1999). Tandem repeats have been identified within the 5' region in multiple digenean families, including Schistosomatidae (Nolan and Cribb, 2005).

Mitochondrial cytochrome c oxidase I. The mitochondrial cytochrome c oxidase I (MCOI) gene is another common choice for investigation of species delimitation and cryptic diversity. In an examination of ITS and mitochondrial sequence data for use in molecular prospecting studies in platyhelminths, Vilas et al. (2005) advocate the use of mitochondrial sequence based on higher rate of evolution, smaller effective population size, and clearer demarcation between inter-specific and intra-specific variation as compared to the ITS. The potential limitations of mitochondrial sequence, including the MCOI, for these studies is intra-specific and intra-individual variation; however, many of the same potential mechanisms for

generating such confounding results must be considered in both ITS and mitochondrial DNA (Vilas et al., 2005). Furthermore, MCOI sequence can be translated to detect pseudogenes, which is not possible for ITS data.

Review of ITS and MCOI in species diversity studies. Ideally, a genetic marker for species identification or assessment of species diversity should have a high level of divergence between closely related species and low intraspecific variation (Vilas et al., 2005). In addition, when examining poorly studied groups, a comparative data set across a broad array of different taxa, digenean trematodes in this case, is helpful for data interpretation and confidence in conclusions. The ITS is a suitable marker for species identification based on evidence for clear interspecific differences between closely related species and low intraspecific variation (Nolan and Cribb, 2005). The MCOI exhibits greater variability than the ITS and is useful for both identifying diagnostic characters and detecting cryptic diversity (Vilas et al., 2005).

In a recent review, Nolan and Cribb (2005) report the use of ITS data in 63 studies involving 19 different families of digeneans. Of these studies, only three reported an absence of differences in ITS sequences between species with strong biological and/or morphological evidence supporting classification as different species (Despres et al., 1992; Blair et al., 1998; Niewiadomska and Laskowski, 2002). A review of sequence data from these studies and additional sequence made available since original publication indicate that the results from two of these studies are suspect (Nolan and Cribb, 2005). Thus, in most instances the ITS regions appear to correlate well with species differences, the only convincing exception being *Paragonimus miyazakii* and *P. skrjabini* (Blair et al., 1998). However, this study only examined the ITS2, which is generally regarded as more conserved than the ITS1. In fact, a minority of studies report the use of the entire ITS, with most using only the shorter ITS2 (Nolan and Cribb,

2005). It is proposed that good study design with regard to the ITS should include the entire ITS in order to increase the ability to discriminate closely related species (Nolan and Cribb, 2005).

The second essential feature of a genetic marker to identify species is low intraspecific variation. Intraspecific variation results in uncertainty regarding which gene variant to use for comparison. Erroneous reports of intraspecific variation are blamed on failure to adequately confirm sequences (sequencing or PCR errors) and failure to recognize multiple species (Nolan and Cribb, 2005). Actual intraspecific variation may occur due to geographic variation or variability in the number of tandem repeats that comprise part of the ITS1 (Nolan and Cribb, 2005). The later is one of the common examples of intraspecific variation observed within the ITS, and is a feature of the ITS1 in some digeneans (Vilas et al., 2005), and may have been overlooked in others. In these instances it is critical to examine the different variants and include other sequences, such as the ITS2 and mitochondrial genes, in any analysis.

Comparison of ITS sequence is a useful method for discriminating digenean trematode species in most instances and has produced compelling evidence for merging synonymous species or more clearly delineating different or cryptic species (Nolan and Cribb, 2005). In some examples, however, intraspecific variation or lack of divergence in ITS sequence between obviously biologically different species has required the use of alternative, more variable genes. These cases serve to illustrate important considerations that must be addressed in studies involving the ITS regions. First, many of the proposed exceptions that argue against the reliability of the ITS involve studies where only the ITS1 or the ITS2 were sequenced. As previously stated, the comparative evidence available across digenean taxa support that both ITS regions should be examined. Second, is the importance of a large sample size and adequate replication to confirm sequence data. These factors lend confidence to results and significance to

minor differences that may otherwise be overlooked or regarded as intraspecific variation. Similarly, supportive data in terms of geographic locality, host species, and other morphological or biological information may be essential.

There are examples that suggest that the ITS may not be the most sensitive indicator of species diversity, at least for some taxa (Nolan and Cribb, 2005). Overlap between observed interspecific and intraspecific distances has been reported in closely related species (Vilas et al., 2005). Mitochondrial genes with greater variability, such as NADH dehydrogenase-1 and cytochrome c oxidase I, may be used as additional supportive data to detect differences in the taxa of interest (Villas et al., 2005). Large-scale studies are needed to better understand and resolve debate over intraspecific variation in the ITS.

In the aforementioned study comparing the use of ITS and mitochondrial sequence for molecular prospecting, Vilas et al. (2005) compared two mitochondrial sequences, the MCOI and NADH dehydrogenase-1 between multiple congeneric species. The major limitation of this study is that it relied heavily on sequences obtained from GenBank, which requires great caution, especially when interpreting small nucleotide differences. Greater divergence between congeneric species was found as compared to ITS, and the authors commented that mitochondrial sequence differing by greater than 5% warrants further investigation of possible species differences.

There are many key differences between mitochondrial DNA and nuclear DNA that have implications on the use of mitochondrial sequence for examining species, i.e. cryptic diversity, and inferring phylogeny. These differences include variability in the degree of recombination, effective population size, mutation rate, and introgression (Ballard and Whitlock, 2004). First, mitochondrial DNA typically does not undergo recombination, except in fungi and plants, thus

the mitochrondrial genome is physically linked (Birky, 2001). As a result, inferences about the history of a population cannot be replicated using other mitochondrial genes. Second, mitochondrial genomes have a smaller effective population size (due to a haploid genome), whereby new alleles are fixed faster than for nuclear DNA. This feature is advantageous for phylogenic studies because it provides faster resolution of coalescence (Ballard and Whitlock, 2004). A third feature of mitochondrial DNA is higher mutation rate, which is dependent in the population size. Small populations tend to accumulate deleterious mitochrondrial alleles, whereas there is greater negative selection in large populations. Actual mutation rates relative to nuclear genes, however, is dependent on the biology of the taxa (Ballard and Whitlock, 2004). Lastly, introgression may be more likely in mitochondrial DNA than nuclear DNA. Introgression is an important consideration in closely related, sympatric taxa due to the probability of hydridization. This process may result in discrepancies in interspecific differences and may be confused with ancestral polymorphism and incomplete lineage sorting in the assessment of gene geniologies (Nielsen and Wakeley, 2001; Ballard and Whitlock, 2004). There are many examples of mitochondrial introgression without nuclear introgression or morphological changes (Ballard and Whitlock, 2004).

Table 1-1. Reported marine spirorchiid trematodes by genus, host species, geographic location, anatomic location, associated pathology, reported prevalence, and citation.

Spirorchid species	Host species	Geographic location	Anatomic location (adults)	Associated Pathology	Prevalence	Reference[a]
Genus *Amphiorchis*						
Amphiorchis amphiorchis	*C. mydas*	Captive	Visceral blood vessels	Not described	N/A	Price, 1934
	E. imbricata	Puerto Rico	Blood vessels of large intestine	Not described	N/A	Fischthal and Acholonu, 1976
Amphiorchis caborojoensis	*E. imbricata*	Puerto Rico	Pulmonary vessels	Not described	N/A	Fischthal and Acholonu, 1976
	E. imbricata	Puerto Rico	Not given	Not described	N/A	Dyer et al. 1995
Amphiorchis indicum	*E. imbricata*	Gulf of Manar, India	"Mesenteric" [sic] capillaries of the intestine			Simha and Chattopadhyaya, 1980[a]
Amphiorchis indicus	*E. imbricata*(?)	Gulf of Manar, India	Heart	Not described	1/3	Gupta and Mehrotra, 1981
Amphiorchis lateralis	*E. imbricata*	Palao Islands				Oguro, 1938[a]
*Amphiorchis (Squaroacetabulum) solus**	*C. mydas*	Gulf of Manar, India	Heart	Not described	N/A	Simha and Chattopadhyaya, 1970
	C. mydas	Tortuguero, Costa Rica	Intestine	Not described	1/40	Santoro et al., 2006
Amphiorchis sp.	*C. mydas*	Australia (western)	Mesenteric arteries	Granulomatous vasculitis, thrombosis, microabscess formation associated with eggs (multiple spirorchid spp. found)	N/A	Raidal et al., 1998
Genus *Carettacola* (*Haemoxenicon*)						
Carettacola bipora	*C. caretta*	Florida, USA	Coelomic blood vessel	Not described	N/A	Manter and Larson, 1950
Carettacola hawaiiensis	*C. mydas*	Hawaii	Hepatic vessels	"Similar findings" to other studies	3/10	Dailey et al., 1991; Dailey et al., 1992
	C. mydas	Hawaii	Not givem	Describe eggs in fibropapillomas	N/A	Dailey and Morris, 1995
	C. mydas	Hawaii	Hepatic vessels	Lymphoplasmacytic endarteritis w/ thrombosis; multi-organ granulomatous inflammation(multiple spirorchid spp. found)	N/A	Graczyk et al., 1995
	C. mydas	Hawaii	Not given	Not described	99/99[d]	Work et al., 2005
Carettacola stunkardi (*C. chelonenecon*)	*C. mydas*	Baja California	Mesenteric veins	Not described	N/A	Martin and Bamberger, 1952

Table 1-1. Continued

Spirorchid species	Host species	Geographic location	Anatomic location (adults)	Associated Pathology	Prevalence	Reference[a]
Carettacola stunkardi (*C. chelonenecon*) (cont.)	*C. mydas*	Panama, Central America				Caballero et al., 1955[a]
Carettacola sp.	*C. caretta*	Atlantic coast, USA	Not given	Some emaciated; multi-organ granulomatous inflammation (multiple spirorchiid spp. Found)	14/43[d]	Wolke et al., 1982
	C. mydas	Australia (western)	Mesenteric veins, liver, aorta	Granulomatous vasculitis, thrombosis, microabscess formation associated with eggs (multiple spirorchid spp. found)	N/A	Raidal et al., 1998
Genus *Cheloneotrema*[b]						
Cheloneotrema testicaudata	*C. mydas*	Gulf of Manar, India	Mesenteric capillaries of intestine			Simha and Chattopadhyaya, 1980[a]
Genus *Hapalotrema*[c] (*Mesogonimus*)						
Hapalotrema pambanensis (*H. mehrai, H. dorsopora*)	*C. mydas*	Gulf of Manar, India	Heart	Not described	N/A	Rao, 1976
	C. mydas	Gulf of Manar, India	Heart	Not described	2/2	Gupta and Mehrotra, 1981
	C. mydas	Hawaii	Heart	Not described	8/10	Dailey et al., 1993
	C. mydas	Hawaii	Not given	Described eggs in fibropapillomas	N/A	Dailey and Morris, 1995
	C. mydas	Hawaii	Heart, major vessels	Lymphoplasmacytic endarteritis w/ thrombosis; multi-organ granulomatous inflammation(multiple spirorchid spp. found)	N/A	Graczyk et al., 1995
	C. mydas	Queensland, Australia	Heart, pulmonary a.	Not described	N/A	Cribb and Gordon, 1998
	C. mydas	Queensland, Australia	Heart, pulmonary a.	Endocarditis/endarteritis; aneurysm formation; thrombosis	91/96[d]	Gordon et al., 1998
	C.mydas	Queensland, Australia	Heart	Not described	N/A	Platt and Blair, 1998
	E. imbricata	Queensland, Australia	Heart	Not described	N/A	Platt and Blair, 1998
Hapalotrema mistroides (H. constrictum, H. loossi)	*C. caretta*	Italy	Aorta (A. celomica) (stated in Looss 1899)			Monticelli, 1896[a]
	C. caretta	Egypt	Heart, major vessels	Vaguely describes endothelial irregularities and vascular thickening	20/21	Looss, 1899; Looss, 1902
	C. caretta	Nile Valley				Gohar, 1934; Gohar, 1935[a]
	C. caretta	Western Australia	Heart, liver	Not described	N/A	Platt and Blair, 1998
	C. mydas	Egypt	Heart, major vessels	Vaguely describes endothelial irregularities and vascular thickening	N/A	Looss, 1902

Table 1-1. Continued

Spirorchid species	Host species	Geographic location	Anatomic location (adults)	Associated Pathology	Prevalence	Reference[a]
Hapalotrema mistroides (H. constrictum, H. loossi) (cont.)	*C. mydas*	Nile Valley				Gohar, 1934; Gohar, 1935[a]
Hapalotrema postorchis	*C. mydas*	Gulf of Manar, India	Heart	Not described	N/A	Rao, 1976
	C. mydas	Hawaii	Heart	Not described	3/10	Dailey et al., 1993
Hapalotrema postorchis (cont.)	*C. mydas*	Torres Strait & Queensland, Australia	Heart, major arteries	Not described	N/A	Platt and Blair, 1998
	C. mydas	Queensland, Australia	Left & right aortas	Endarteritis; aneurysm formation; thrombosis	91/96[d]	Gordon et al., 1998
	C. mydas	Queensland, Australia	Left & right aortas	Not described	N/A	Cribb and Gordon, 1998
	C. mydas	Tortuguero, Costa Rica	Heart, great vessels	Not described	8/40	Santoro et al., 2006
Hapalotrema synorchis (*H. orientalis*)	*C. caretta*	Florida, USA	Heart	Not described	1/3	Luhman, 1935
	C. caretta	Western Australia & Queensland	Heart	Not described	N/A	Platt and Blair, 1998
	E. imbricata	Okinawa, Japan				Takeuti, 1942[a]
	E. imbricata	Puerto Rico	Heart	Not described	N/A	Fischthal and Acholonu, 1976
Hapalotrema sp.	*C. caretta*	Atlantic coast, USA	Not given	Some emaciated; multi-organ granulomatous inflammation (multiple spirorchiid spp. Found)	14/43[d]	Wolke et al., 1982
	C. mydas	Australia (northern)	Heart, major arteries	Endcarditis; endarteritis w/ thrombosis; Multi-organ granulomatous inflammation	17/22 (wild only) [d]	Glazebrook et al., 1981 and 1989
	C. mydas	Australia (northern)	Heart	"[T]hickening and hardening of arterial walls with thrombus formation"; generalized emaciation; chronic pneumonia	16/21 (wild only)[d,e]	Glazebrook and Campbell,1990
	C. mydas	Hawaii	Heart		8/10 [d]	Dailey et al., 1992
	C. mydas	Queensland, Australia				Barker and Blair, 1996
	C. mydas	Queensland, Australia	Liver/lung wash	Not described	N/A	Platt and Blair, 1998
	C. mydas	Australia (western)	Aortas	Granulomatous vasculitis, thrombosis, microabscess formation associated with eggs (multiple spirorchid spp. Found)	N/A	Raidal et al., 1998
	C. mydas	Hawaii	Not given	Not described	99/99 [d]	Work et al., 2005
	E. imbricata	Australia (northern)		Endcarditis; endarteritis w/ thrombosis; Multi-organ granulomatous inflammation	17/22 (wild only) [d]	Glazebrook et al., 1989
	E. imbricata	Australia (northern)	Not specified	"[T]hickening and hardening of arterial walls with thrombus formation"; generalized emaciation; chronic pneumonia	1/1 (wild only) [d,e]	Glazebrook and Campbell, 1990

Table 1-1. Continued

Spirorchid species	Host species	Geographic location	Anatomic location (adults)	Associated Pathology	Prevalence	Reference[a]
Genus *Learedius*						
Learedius learedi	*C. mydas*	Captive	Circulatory system	Not described	N/A	Price, 1934
	C. mydas	Panama				Caballero, et al., 1955[a]
	C. mydas	Gulf of Manar, India		Gulf of Manar, India		Gupta and Mehrotra 1975[a]
	C. mydas	Australia	Heart	Australia		Blair, 1979 (quoted by Glazebrook et al., 1989)
	C. mydas	Grand Cayman Island	Heart, major arteries			Greiner et al., 1980
	C. mydas	Bermuda	Heart	Multi-organ granulomatous inflammation	11/11	Rand and Wiles, 1985
	C. mydas	Puerto Rico	Heart	Not described	N/A	Dyer et al., 1991
	C. mydas	Puerto Rico				Williams et al,. 1994
	C. mydas	Hawaii	Heart	Not described	4/10	Dailey et al., 1992 and 1993
	C. mydas	Hawaii	Not given	Describe eggs in fibropapillomas	N/A	Dailey and Morris, 1995
	C. mydas	Hawaii	Heart, major vessels	Endarteritis, thrombi formation, multi-organ granulomatous inflammation (multiple spirorchid spp. found)	N/A	Graczyk et al., 1995
	C. mydas	Tortugeuro, Costa Rica	Heart, major vessels, other sites	Not described	39/40	Santoro et al., 2006
	C. agassizii	Baja California	Heart	Multi-organ granulomatous inflammation	11/11	Cordero-Tapia et al., 2004
	E. imbricata	Puerto Rico	Heart	Not described	N/A	Dyer et al., 1995
Learedius loochooensis	*C. mydas*	Japan				Takeuti, 1942[a]
Learedius orientalis (possibily syn. w/ *L. learedi*)	*C. mydas*	Arabian Sea	Heart	Arabian Sea		Mehra ,1939[a]
Learedius orientalis (possibily syn. w/ *L. learedi*) (cont.)	*C. mydas*	Gulf of Manar, India	Heart	Not described	2/2	Gupta and Mehrotra, 1975[a]
	C. mydas	Puerto Rico	Heart, aorta	Not described	1/4	Dyer et al., 1995
	E. imbricata	Puerto Rico	Heart	Not described	3/14	Fischthal and Acholonu, 1976
Learedius similis	*C. mydas*	Captive	Circulatory system	Not described	N/A	Price, 1934

Table 1-1. Continued

Spirorchid species	Host species	Geographic location	Anatomic location (adults)	Associated Pathology	Prevalence	Reference[a]
Learedius sp.	*C. mydas*	Australia (northern)	Heart, right aortic arch, brachiocephalic artery	"[T]hickening and hardening of arterial walls with thrombus formation"; generalized emaciation; chronic pneumonia	16/21 (wild only) [d]	Glazebrook and Campbell, 1990
	C. mydas	Australia (northern)	Heart, major arteries	Endcarditis; endarteritis w/ thrombosis; Multi-organ granulomatous inflammation	17/22 (wild) [d]	Glazebrook et al., 1989
Learedius sp. (cont.)	*C. mydas*	Hawaii	Not given	Not described	99/99 [d]	Work et al,. 2005
	E. imbricata	Australia (northern)	Heart, major arteries	Endocarditis; endarteritis w/ thrombosis; Multi-organ granulomatous inflammation	17/22 (wild only) [d]	Glazebrook et al., 1989
Genus *Monticellius*						
Monticellius indicum	*C. mydas*	Arabian Sea	Heart			Mehra, 1939[a]
	C. mydas	Tortuguero, Costa Rica	Heart	Not described	5/40	Santuro et al., 2006
Genus *Neospirorchis*						
Neospirorchis pricei	*C. caretta*	Florida, USA	Heart	Not described	N/A	Manter and Larson, 1950
Neospirorchis schistosomatoides	*C. mydas*	Captive	Visceral blood vessels	Not described	N/A	Price, 1934
	C. mydas	Bermuda	Heart	Multi-organ granulomatous inflammation	2/11 (6/11 had *Neosp*. eggs)	Rand and Wiles, 1985
	C. mydas	Queensland, Australia	Meningeal venules	Granulomatous inflammation	91/96[d]	Gordon et al., 1998
Neospirorchis sp.	*C. caretta*	Atlantic coast, USA	Not given	Some emaciated; multi-organ granulomatous inflammation (multiple spirorchiid spp. found)	14/43[d]	Wolke et al., 1982
	C. caretta	Florida, USA	Meningeal venules	Granulomatous inflammation		Jacobson et al., 2007
Genus *Neocaballerotrema*						
Neocaballerotrema caballeroi	*C. caretta*	Gulf of Manar, India	Enteric blood vessel			Simha, 1977[a]
Genus *Satyanayanotrema*[a]						
Satyanayanotrema satyanarayani	*C. mydas*	Gulf of Manar, India	"Blood vessel"			Simha and Chattopadhyaya, 1980[a]
Genus *Shobanatrema*						
Shobanatrema shobanae	*C. caretta*	Gulf of Manar, India	"Mesenteric [sic] capillaries of large intestine"			Simha and Chattopadhyaya, 1980[a]

[a]Reference unavailable, cited in Smith 1997; [b]Multiple spellings; [c]Based redescriptions of species in the genus *Hapalotrema* by Platt & Blair (1998), Cribb & Gordon (1998), and Platt (2002). Junior synonyms are given in parentheses; [d]Pravelence data includes multiple spirorchiid genera or species; [e]Prevalence data includes multiple sea turtle species; *Platt 2002 placed as junior synonym of *Amphiorchis*

Table 1-2. Marine spirorchiid trematodes by turtle definitive host species with locality and reference citation.

Host species	Spirorchid species	Locality	Reference
C. mydas			
	Amphiorchis amphiorchis	Captive	Price ,1934
	Amphiorchis sp.	Australia (western)	Raidal et al.., 1998
	Carettacola hawaiiensis	Hawaii	Dailey et al., 1991 and 1993; Dailey and Moris 1995; Graczyk et al., 1995; Work et al., 2005
	Carettacola stunkardi	Baja California	Martin and Bamberger, 1952; Cabellero et al., 1955
	Carettacola sp.	Australia (western)	Raidal et al., 1998
	Cheloneotrema testicaudata	India	Simha and Chattopadhyaya, 1980
	Hapalotrema pambanensis	India	Rao 1976; Gupta and Mehrotra 1981
		Hawaii	Dailey et al., 1993; Dailey and Morris, 1995; Garczyk et al., 1995
		Queensland, Australia	Cribb and Gordon, 1998; Gordon et al., 1998; Platt and Blair, 1998
	Hapalotrema mistroides	Egypt	Looss, 1902
	Hapalotrema postorchis	Egypt	Rao, 1976
		Hawaii	Dailey et al., 1993
		Australia	Platt and Blair, 1998, Gordon et al.. 1998; Cribb and Gordon, 1998
	Hapalotrema sp.	Australia	Glazebrook et al., 1981 and1989; Glazebrook and Campell, 1990; Barker and Blair, 1996; Platt and Blair, 1998; Raidal et al., 1998
		Hawaii	Dailey et al., 1992; Work et al., 2005
	Learedius learedi	Captive	Price, 1934
		Panama	Caballero et al., 1955
		India	Gupta and Mehrotra, 1975
		Australia	Blair ,1979
		Caribbean	Greiner et al., 1980, Dyer et al., 1991; Williams et al., 1994
		Bermuda	Rand and Wiles, 1985
		Hawaii	Dailey et al., 1992 and 1993: Dailey and Morris, 1995; Graczyk et al., 1995;
		Baja, California	Cordero-Tapia et al., 2004
	Learedius loochooensis	Japan	Takeuti, 1942
	Learedius orientalis	Arabian Sea	Mehra, 1939
		India	Gupta and Mehrotra, 1976
		Puerto Rico	Dyer et al., 1995
	Learedius similis	Captive	Price, 1934
	Learedius sp.	Australia	Glazebrook & Campbell 1990; Campel et al., 1989
		Hawaii	Wolk et al. 2005
	Montecellius indicum	Arabian Sea	Mehra 1939
	Neospirorchis schistosomatoides	Captive	Price 1934
		Bermuda	Rand and Wiles, 1985
		Australia	Gordon et al., 1998

Table 1-2. Continued

Host species	Spirorchid species	Locality	Reference
C. mydas (cont.)	*Satyanayanotrema satyanarayani*	India	Simha and Chattopadhyaya ,1980
	Squaroacetabulum solus	India	Simha and Chattopadhyaya ,1970
C. caretta			
	Carettacola bipora	Florida	Manter and Larson, 1950
	Carettacola sp.	Atlantic coast, USA	Wolke et al., 1982
	Hapalotrema mistroides	Italy	Monticelli, 1896
		Egypt	Looss, 1899 and 1902; Gohar, 1934 and 1935
		Australia	Platt and Blair, 1998
	Hapalotrema synorchis	Florida, USA	Luhman, 1935
		Australia	Platt and Blair, 1998
	Hapalotrema sp.	Atlantic coast, USA	Wolke et al., 1982
	Neospirorchis pricei	Florida, USA	Manter and Larson, 1950
	Neospirorchis sp.	Atlantic coast, FL	Wolke et al., 1982
		Florida, USA	Jacobson et al., 2007
	Neocaballerotrema caballeroi	India	Simha, 1977
	Shobanatrema shobanae	India	Simha and Chattopadhyaya, 1970
E. imbricata			
	Amphiochis amphiorchis	Puerto Rico	Fischthal and Acholonu, 1976
	Amphiorchis caborojoensis	Puerto Rico	Fischthal and Acholonu, 1976; Dyer et al., 1995
	Amphiorchis indicum	India	Simha and Chattopadhyaya, 1980
	*Amphiorchis indicus**	Palao Islands	Oguro, 1938
	Hapalotrema pambanensis	Australia	Platt and Blair, 1998
	Hapalotrema synorchis	Japan	Takeuti, 1942
		Puerto Rico	Fischthal and Acholonu, 1976
	Hapalotrema sp.	Australia	Glazebrook et al., 1989; Glazebrook and Campbell, 1990
	Learedius learedi	Puerto Rico	Dyer et al., 1995
	Learedius orientalis	Puerto Rico	Fischthal and Acholonu, 1976
	Learedius sp.	Australia	Glazebrook et al., 1989

CHAPTER 2
SPIRORCHIID TREMATODES OF STRANDED SEA TURTLES IN FLORIDA AND ASSOCIATED PATHOLOGY

Introduction

Florida and its coastal waters are critical foraging and nesting habitat for the Atlantic loggerhead turtles (*Caretta caretta*) and Caribbean-Atlantic green turtles (*Chelonia mydas*). Approximately 1,200 to 1,500 strandings are documented annually in Florida, which far surpasses other Atlantic and Gulf regions monitored under the Sea Turtle Stranding and Salvage Network (STSSN). Health-related studies in Florida sea turtles have included investigations of fibropapillomatosis, brevetoxicosis, hypothermic (cold)-stunning, and other unusual mortality events (Witherington and Erhart, 1989; Herbst et al., 1994; Foley et al., 2005; Jacobson et al., 2006). These studies have provided insight into important health issues, but also have revealed fundamental gaps in our knowledge of sea turtle diseases. Spirorchiid trematode infections in particular have been a recurring health issue of concern in Florida sea turtles largely due to observations of heavily infected individuals in the stranding population and an uncertain role in at least one unusual mortality event (Wolke et al., 1982; Jacobson et al., 2006).

Sea turtles serve as definitive hosts for approximately twenty-four different described species of spirorchiid trematodes representing ten genera (Smith, 1997b). These numbers almost certainly are an under representation of actual parasite diversity as turtles inhabiting many geographical regions and some species have yet to be thoroughly examined. Studies of marine spirorchiids tend to be polarized as formal morphological descriptions and surveys of parasites, or descriptions of associated host pathology. Very few investigators have effectively combined specific identification of spirorchiid species, characterization of host pathology, and interpretation of significance in the context of complete necropsies. Furthermore, previous studies have been heavily biased towards chronically ill turtles, which often are diagnostically

complex and require heavily qualified or conservative interpretation (Wolke et al., 1982, Gordon et al., 1998). As a result, the picture of spirorchiids and their impact on sea turtle health is somewhat distorted and very incomplete. Basic information, such as prevalence of spirorchiids and associated pathological lesions in stranded turtles, is sparse or completely lacking in many regions, including Florida.

The aim of these studies was to assess the significance of spirorchiidiasis and characterize associated disease in stranded *C. caretta* and *C. mydas* by examining the following factors: impact of spirorchiids as a cause of death or contributing cause of death; diversity and prevalence of spirorchiids, microhabitat utilization in the turtle host, and associated pathological lesions; and relationships between parasitism and duration of illness and host age/size class. These various facets were selected to address critical unknown information relevant to Florida's stranded sea turtles, as well as health implications in sea turtles worldwide. The resulting findings provide the first comprehensive assessment of spirorchiidiasis in Florida turtles and new insight into host-parasite-disease relationships.

Materials and Methods

Necropsies and Parasite Collection

Necropsies were performed on stranded *C. caretta* and *C. mydas* recovered in Florida by the Sea Turtle Stranding and Salvage Network from November 2004 through October 2007. Two stranded turtles from Georgia (Glynn and Chatham counties) also were examined. The study was limited to wild turtles that were found dead, died soon after discovery, or that were in rehabilitation centers for 10 days or less. Four categories of postmortem examination were performed depending on circumstances, postmortem condition, and whether or not the carcass had been previously frozen. Category 1 necropsies included gross and histopathological examination of all organs and tissues, and complete parasitological methods for detection of

spirorchiid trematodes. Category 2 necropsies included gross assessment, complete parasitological methods for detection of spirorchiid trematodes, and, in some cases, limited histopathology of major lesions. Category 1 and 2 necropsies were performed at the University of Florida, College of Veterinary Medicine. Category 3 necropsies consisted of gross necropsy and evaluation of target organs and sites for spirorchiid trematodes (see parasitological methods) and associated lesions. These necropsies primarily were performed at the Florida Fish and Wildlife Conservation Commission (FWCC) Marine Mammal Pathology Laboratory in St. Petersburg, Florida. Category 4 examinations were limited to evaluation of the brain and meninges for the presence of spirorchiid trematodes. Categories 2 through 4 generally were previously frozen and/or autolyzed carcasses.

Parasitological Methods

The primary objective of the parasitological methods performed during necropsy was to detect and recover spirorchiid trematodes. These methods included a combination of gross examination, examination target organs using a dissecting microscope, screening of body fluids and organ washes, and histopathology. During the course of the necropsy, the liver, body cavity, heart (and base of aortas and pulmonary arteries), aorta (left, right and dorsal), and mesenteric arteries were examined and vascular compartments were thoroughly washed and filtered using a #45 sieve. The filtrate was examined for the presence of spirorchiids. In addition, the brain, heart, any vascular lesions, thyroid gland, thymus, adrenal glands, urinary bladder, and sections of stomach and intestine were examined using a dissecting microscope for the presence of spirorchiid eggs and adults. For category 3 necropsies, the complete aorta was evaluated for areas of endarteritis and the brain, thyroid gland, adrenal glands, and thymus were examined under a dissecting microscope. Category 4 necropsies were limited to examination of the brain using a dissecting microscope. Identification of spirorchiids was based on examination of adults

whenever possible. When only eggs were detected, morphology alone was used to identify the presence of the genera *Neospirorchis* and *Carettacola*; however, egg morphology was not used to distinguish *Hapalotrema* and *Learedius* egg types in green turtles. In such cases, the presence of eggs with bipolar processes (Type I) was noted, but neither generic nor specific identification was made. Lastly, observed spirorchiid eggs, especially *Neospirorchis* sp., in tissues were classified as primary site oviposition verses embolization to distant locations. Primary sites of oviposition were characterized by the presence of large and/or discrete egg masses (typically greater than 100 eggs) and often were observed in the presence of adult spirorchiids. Embolized eggs most commonly were diffusely distributed throughout a tissue or organ and were either individual eggs or small clusters of less than ten.

Laboratory Methods

Samples for histological examination were fixed in 10% neutral phosphate buffered formalin and processed by routine methods into paraffin blocks, which were cut into 5 μm-thick sections and stained with hematoxylin and eosin. Spirorchiid trematodes were fixed in alcohol-formalin-acetic acid (AFA) if intact or nearly complete and representative specimens (including adults and eggs) were frozen at -80º Celsius.

Categorization of Duration of Illness

Necropsied turtles were placed into three categories according to the estimated duration of illness. The subjective criteria for classification included both nutritional condition and necropsy findings (Table 2-1). Nutritional condition was the primary determining factor and was assessed by evaluation of skeletal muscle, adipose tissue, skeletal mineralization, and presence or absence of a generalized catabolic state. The secondary factor is the apparent duration of any identifiable fatal or contributory pathological lesion. Assessment of lesion chronicity was based on standard gross and histopathological interpretation, e.g. primarily granulocytic inflammation

and fibrinous exudation indicate acute processes and predominantly mononuclear inflammation, granulation tissue formation and fibrosis are indicators of chronicity. The actual time scale of these categories is a crude estimate as the progression of weight loss in sea turtles has not been studied and many factors may influence the progression of disease, such as severity of insult, degree of debilitation, and environmental parameters. Acute insults include immediate fatality and animals that were estimated to be ill on the order of days to weeks. Turtles with intermediate insults were estimated to have been ill for weeks to months. Those animals categorized under chronic insult likely have been ill on the order of months or longer.

Primary Diagnosis / Cause of Death Designations

Necropsied turtles also were categorized by the primary diagnosis or cause of death. Categories included traumatic injury, brevetoxicosis, hypothermic (cold-stunning), mass mortality events (MME) of unknown etiology, drowning/aspiration, infectious disease, enteric impaction, fibropapillomatosis-associated, emaciation, multifactorial, and undetermined. These designations were given to represent the primary health problem, if determinable. For example, a turtle that died of secondary bacterial infection of a boat propeller wound was categorized as a traumatic injury. Similarly, animals that ultimately drown from underlying disease were categorized by the nature of the underlying disease, if identified. Primary drowning/aspiration cases were diagnoses by exclusion, often were accompanied by circumstantial evidence (e.g. recovered from trawler net), and were not associated with recognized MME's. Brevetoxicosis, unsolved MME's, and hypothermic deaths were supported by epidemiological stranding data, environmental data, and supportive diagnostics from the investigations of these events, rather than specific pathological findings. Emaciation was listed as the primary diagnosis if poor nutritional condition was the only necropsy finding of significance. Deaths were ruled multifactorial if multiple processes were present and were interpreted to have contributed to

death. In every use, this designation reflected multiple health problems in chronically ill, emaciated turtles.

Spirorchiid Impact Rating

Spirorchiid trematode infection and associated pathological lesions was evaluated based on intensity of spirorchiid infection, severity of lesions, proportion of organ involvement, and the organ or tissue affected. All data were assessed in the context of other necropsy findings and cause of death, if determined, and scored into one of five categories. The criteria for scoring are given in Table 2-2. Intensity of parasitism classically is measured by quantifying adult parasites and or eggs, e.g. fecal egg counts. With regard to spirorchiid trematodes, the numbers of adult parasites often do not appear to correlate with severity of pathological lesions and the intravascular location and small size of adults makes confident quantification extremely difficult or impossible (Gordon et al., 1998). Also, the variety of tissues in which eggs selectively accumulate makes any objective measure that would be comparable between cases very difficult. Therefore, the intensity of spirorchiidiasis in this study is based on a relative assessment of absent, rare, small, moderate, or large numbers, reflecting both adult and egg numbers within various organs and tissues as observed at the gross and subgross levels. Photographs of cases and postmortem data were then retrospectively compared to support that abundance ratings were consistent within categories. In addition, specific grading criteria were created for vascular, neurological, thyroid/thymic, and gastrointestinal lesions (Table 2-3). A spirorchiid impact rating was only applied to category 1 and 2 necropsies.

Body Condition Calculations for Immature *C. mydas*

Error in body weight measurements due to variation between equipment, epibiota accumulation, and severe traumatic injuries prevented confident assessment of body condition indices in the general study population. A group of immature *C. mydas* that died from

hypothermic-stunning did not have these confounding problems, thus additional body condition data were collected. Two body condition indices were calculated, a simple mass to length ratio (Body mass [BM] [kg]/Straight carapace length [SCL][cm]) and estimated volume ratio (BM/SCL^3), which has been used in previous sea turtle studies (Bjorndal et al., 2000; McMichael, 2005; Work et al., 2005;).

Statistical Methods

Comparisons were made between various aspects of spirorchiidiasis, including presence/absence, pathological lesions, and grade, in turtles categorized by duration of illness (see previous criteria) and size. Three size classes used were based on straight carapace and included SCL ≤65 cm, >65-85 cm, and >85cm. Some factors were combined to support statistical analysis when appropriate. Parameters were compared by Pearson's chi square or Fisher exact test (95% confidence interval), depending on the expected frequencies, using Analyse-it® version 2.11. Also examined were differences in body condition indices in immature *C. mydas* with and without moderate or large numbers of embolized spirorchiid eggs and intra-thyroid *Neospirorchis* species using the Mann-Whitney test.

Results

Caretta caretta

Study population demographics, duration of illness categories

A total of eighty-nine *C. caretta* were necropsied. The number of *C. caretta* in each necropsy data category by geographical region is shown in Figure 2-1. The frequency distribution of turtle sizes had a bimodal distribution with peak frequencies in the 60-75 cm SCL and 85-95 cm SCL size ranges (Figure 2-2). All turtles >85 cm SCL were confirmed to be sexually mature. The female to male sex ratio was strongly biased at 3.94:1 (71/18). Category 1 and 2 data included 54 turtles, 20 in the acute insult category, 14 in the intermediate duration

category, and 20 in the chronic insult category (Table 2-4). The size class compositions of the duration of illness categories were compared. The only statistical difference was that there was a higher proportion of turtles (category 1 & 2 data) greater than 85 cm SCL in the acutely affected group as compared to the those with chronic insults ($p<0.05$). The same bias was observed when all necropsy data categories (1-4) were analyzed ($p<0.005$). The primary reason for this bias was traumatic deaths of adults during breeding and nesting season.

Primary diagnosis or cause of death by duration of illness

Acute insult. The primary diagnoses or causes of death of *C. caretta* that died from acute insults included traumatic injury (6/20), brevetoxicosis (7/20), and deaths associated with a mass mortality event of unknown etiology (4/20) (Table 2-4). Traumatic injuries were due to watercraft collisions (2/6), injuries resulting from a fall (from a sea wall) (1/6), shark attack (1/6), and a probable hooking injury (1/6). One turtle died of a unilateral coracoid fracture of unknown cause that resulted in secondary perforation of the left aorta. All brevetoxicosis cases were associated with a *Karenia brevis* bloom in southwestern Florida (Zone 2) in 2005. The turtles that died associated with a mass mortality event of unknown etiology were all from the same event, which was documented in northeastern Florida and Georgia coasts (Zone 3) in 2006. Some type of acute toxicosis was suspected based on the lack of any evidence of primary infectious disease, traumatic injuries, circumstantial environmental data, or other apparent cause. Testing for brevetoxin, ciguatoxin, saxitoxin, palytoxin, and domoic acid were all negative (data not shown). Of the remaining three turtles in the acute insult category, one drown in a trawler fishing net (1/20), one died of aspiration pneumonia due to unknown circumstances (1/20), and the cause of death of a third case could not be determined (1/20).

Insult of intermediate duration. Necropsy cases categorized in the intermediate duration category included deaths associated with the aforementioned mass mortality event of unknown

etiology in 2006 (6/14), inflammatory/infectious disease (5/14), traumatic injuries (2/14), and one turtle that died from an enteric impaction associated with severe colitis (1/14) (Table 2-4). Deaths associated with infectious disease or inflammatory conditions were due to a variety of causes and affected systems. Cases included septic cholecystitis (1/5), fungal tracheobronchitis (1/5), chronic pneumonia of unknown etiology (1/5), necrotizing dermatitis (1/5), and septicemia with thromboembolic disease (1/5). The case of thromboembolic disease had atrial thrombi that formed in association with areas of intense endocardial spirorchiid egg deposition, which was interpreted as predisposing or confounding condition. The two deaths resulting from traumatic injuries were both cases with chronic propeller wounds that entered the spinal canal and resulted in severe ascending bacterial meningitis (2/14).

Chronic insults. Most postmortem findings in chronically ill *C. caretta* were regarded as either nonspecific lesions associated with chronic illness and advanced poor nutritional status or opportunistic infections. Interpretation of some pathological findings as opportunistic (secondary) infections was based on the relatively acute nature of the inflammatory response (predominantly granulocytic) as compared to advanced poor nutritional status/chronic illness. Some chronic conditions also were identified, but only in a single case (discussed below) could these findings be argued to be an inciting or primary cause of general illness. Nonetheless, these processes may have ultimately contributed to death in many cases or exacerbated decline in clinical status.

Most of the necropsied *C. caretta* categorized under chronic disease had multiple disease processes of significance and cause of death was classified as multifactorial (13/20) (Table 2-4). No underlying primary cause of disease could be confidently identified in these cases, nor was there any apparent temporal or other association with known mass mortality events. In addition

to being profoundly emaciated, common diagnoses interpreted to be contributory to death in these animals included ulcerative gastritis (8/13), ulcerative enteritis and/or colitis (7/13), dermatitis (most often associated with accumulation of epibiota) (8/13), granulomatous hepatitis (4/13), and cutaneous and/or musculoskeletal trauma associated with advanced catabolic state (2/13). In fewer cases, emaciation alone was the primary significant finding (3/20).

In 4/20 cases, a single or predominant process was evident as an underlying condition or proximate cause of death. One turtle had severe chronic cardiovascular and gastroenteric spirorchiid-associated lesions that were interpreted as the cause of death. Two turtles had severe chronic colon impactions with associated ulcerative colitis. Lastly, one turtle had chronic, obstructive inflammation of all major bile ducts (choledochitis) and drowned after entering a coastal power plant facility.

Prevalence by genus and identifiable species (category 1 & 2 data).

Spirorchiids detected in the study population were species within the genera *Neospirorchis*, *Hapalotrema*, and *Carettacola*. Period prevalence of *Neospirorchis* sp. was 96.3% (52/54), prevalence of *Hapalotrema* sp. was 77.8% (42/54), and prevalence of *Carettacola* sp. was 22.2% (12/54). All adult *Carettacola* were identified as *C. bipora*. Mixed infections by all three genera were observed in 11/54 infected turtles, two genera in 32/54 turtles, and single species infections were detected in only 9/54 turtles.

Three species of *Hapalotrema* were found in *C. caretta*. These included *H. mistroides*, *H. pambanensis*, and a novel species (Figure 2-3). Identification of species was performed in 20/25 cases in which adults were collected. *Hapalotrema mistroides* was identified in 17/20 cases, *H. pambanensis* in 5/20 cases, and the novel *Hapalotrema* sp. in 2/20 cases.

Specific species identification by morphology was not possible for most of the recovered *Neospirorchis* species because intact specimens suitable for identification were extremely

difficult to collect. A diagnosis of *Neospirorchis* was based on egg morphology, elongate body type, and examination of anterior and posterior ends of some fragmented specimens. The only exception was *N. pricei*, which was easily recovered intact from the heart and major arteries. Prevalence of this species was 11.1% (6/54).

Site predilection and associated pathological lesions

***Hapalotrema* species.** Species within the genus *Hapalotrema* were observed in the heart, aortas, mesenteric arteries and hepatic vessels, and pathological lesions in major arteries were seen in 37.2% (29/78) of *C. caretta* in category 1-3 necropsies (Table 2-5). Of those parasites identified to the species level, the sites from which adult spirorchiids were recovered are given in Table 2-6. When categorized by duration of illness (category 1 & 2 necropsies), there were no detectable differences in numbers of infected turtles. There were, however, differences when status of infection was compared by size class. There were more infected turtles in the >85 cm SCL group than the ≤65 cm SCL group ($p < 0.05$).

Lesions of major arteries, including the aortas and major branches were associated with *Hapalotrema* species based on the presence of adults attached within areas of arteritis in representative cases. Only one case had endarteritis with no evidence of *Hapalotrema* infection. Lesions in this case were minimal and chronic, which may reflect a resolved or mild infection. The discovery of multiple *Hapalotrema* species in *C. caretta* somewhat complicates association of a specific species with pathologic lesions because four of 20 turtles in which *Hapalotrema* species were identified had mixed infections including *H. mistroides* and *H. pambanensis*. However, only two turtles with mixed infections had major arterial disease, and *H. mistroides* was the only species identified in the remainder of cases from which adults were recovered. Also, neither of the two cases infected with the novel *Hapalotrema* species had arterial disease affecting larger arteries.

In category 1 and 2 necropsies, the most common site of endarteritis/arteritis was the left aorta (n=25), followed by the superior mesenteric artery (n=11), right aorta (n=4), gastric artery (n=3), coeliac artery (n=1), and dorsal aorta (n=1) (Figure 2-4). Multiple vessels were affected in 9/29 cases. Gross lesions ranged from single small plaques of endarteritis to larger areas of arteritis with associated generalized thickening of the artery wall (3 to 4 mm) involving the entire left aorta and, in some cases, the superior mesenteric, coeliac, and gastric arteries. Mural thrombi were observed in 10 cases and all were non-occlusive. Aneurysm formation was present in a single turtle at the proximal superior mesenteric artery. Major arterial lesions were histologically examined in category one necropsies (n=24). Mild lesions (Grade 1) were characterized by mild subintimal fibrosis and a predominantly mononuclear inflammatory infiltrate. More severe lesions included variably intense pleocellular infiltrate, fibromuscular proliferation (often villus) of the intima (resulting in severe thickening of the artery wall), and degeneration and necrosis of the tunica media (Figure 2-5). The latter was a distinctive lesion characterized by formation of well-demarcated necrotic areas of smooth muscle surrounded and infiltrated by heterophils (Figure 2-6). This lesion was observed in seven cases. Inflammation extended transmurally in fifteen cases and often formed patchy or diffuse cuffs of intense pleocellular infiltrate within the adventitia (Figure 2-5). Granuloma formation within lesions was common and most often included intralesional spirorchiid eggs, necrotic debris, and pigmented material.

No significant differences were detected in the presence of arteritis or grade of arteritis when turtles were categorized by duration of illness. There were, however, significant differences based on turtle size. More large turtles (>85 cm SCL) had endarteritis than either the >65-85 cm or ≤65 cm SCL categories ($p<0.005$ and $p<0.0001$, respectively). There were no

detectable significant differences in grade of arteritis among infected turtles of the different size groups.

Pathological lesions affecting small arteries were histologically observed in gastrointestinal tract and associated other organs in eleven turtles, all of which were infected by *Hapalotrema* species. Observed lesions included endarteritis, medial hypertrophy, microvascular proliferation, and periarteritis (Figure 2-7). Organs/sites with affected vessels included the gastrointestinal tract (n=9), pancreas (n=2), kidney (n=1), and adrenal gland (n=1). All but two turtles had concurrent endarteritis of major vessels and 6/9 had grade 3 lesions. Some turtles had grossly visible *Hapalotrema* egg masses within subserosal vessels. Two distinct gross appearances of these egg masses were observed, the most common of which was small, raised, brown masses two to three millimeters in diameter (Figure 2-8). Most (6/7) of these turtles with observable emboli had grade 2 or 3 lesions in major arteries and were infected with *H. mistroides* or had mixed (*H. mistroides* and *H. pambanensis*) infections. The second gross lesion type was represented by a single case with large transmural egg granulomas visible from the mucosal and serosal surfaces (Figure 2-9). This turtle did not have major arterial disease and was infected by the novel *Hapalotrema* species. Adults and egg emboli were limited to the smaller arteries.

Six *C. caretta* also had vasculitis of hepatic portal veins. All cases were infected with *Hapalotrema* sp., but no adults were recovered from the hepatic vasculature in any case. Two turtles also were infected with *C. bipora*, and two adults were removed from the liver vasculature in one of these animals, thus it is possible that *C. bipora* is a cause of this lesion in some cases.

Additional lesions associated with infection by *Hapalotrema* sp. included two cases with papillary lesions associated with submucosal granulomatous inflammation in response to

spirorchiid eggs. The first was the formation of colonic polyps in a turtle infected by *H. mistroides* (Figure 2-10). The second case had severe granulomatous cholecystitis with papillary mucosal hyperplasia associated with the novel *Hapalotrema* species (Figure 2-11).

***Neospirorchis* species.** Spirorchiid eggs consistent in morphology with the genus *Neospirorchis* were widely embolized throughout the body. Dense concentrations of eggs, often associated with adult parasites, were consistently found in several anatomic locations including the vasculature of the leptomeninges, endocrine organs, thymus, and submucosa of the alimentary tract. The period prevalence of *Neospirorchis* species observed in these locations for all necropsy categories is given in Table 2-7.

***Neospirorchis* sp.: central nervous system.** Adult *Neospirorchis* sp. and/or egg masses were observed in the leptomeninges of almost half (44/89) of the necropsied stranded *C. caretta* (all categories included) (Table 2-8) (Figure 2-12). The majority (29/45) of these cases were grade 1 infections, although, more than ten percent were classified as grade 3. There were no significant differences detected in either the proportions of infected turtles or grade of infection when the study population was categorized by duration of illness. There were, however, differences when turtles were compared by size. A significantly higher proportion of turtles >85 cm SCL were infected as compared to turtles between >65- 85 cm SCL ($p < 0.01$) and turtles $\leq$65 cm SCL ($p < 0.0001$). There were no demonstrable significant differences in the grade among infected turtles between the size categories. However, very few infected turtles were $\leq$65 cm SCL (n=5), thus more confident assessment would require a larger sample size.

Adult trematodes and egg masses were most commonly within venules of the leptomeninges and surface vessels of the brain and spinal cord. Individual eggs and egg masses were both intravascular and perivascular. There was minimal or no inflammatory response

associated with adult trematodes. Egg masses often were surrounded by granuloma formation, including intravascular granuloma formation, and a predominantly mononuclear infiltrate, resulting in meningitis and choroiditis. The severity of inflammation, with regard to distribution and intensity, corresponded to the number and distribution of eggs present in most cases. A fibrin thrombus was observed in a single case with grade 3 infection. No associated ischemic or hemorrhagic lesions affecting the brain or spinal cord were observed in any infected turtles.

***Neospirorchis* sp.: endocrine organs.** The endocrine organ in which spirorchiid infection was most commonly observed was the thyroid gland (Figure 2-13). Of category 1, 2, and 3 turtles, 54.6% (41/75) had adult *Neospirorchis* sp. and/or eggs within the thyroid gland and 22.0% (9/41) of those infected were classified as grade 3 (Table 2-9). Of the category 1 and 2 necropsies, 44.4% (24/54) had adult spirorchiids observed within the thyroid gland. When categorized by duration of illness, significantly more chronically ill turtles were infected as compared to animals in the acute ($p<0.005$) and intermediate duration categories ($p <0.05$). Also, proportionately more chronically ill turtles had grade 3 infections as compared to acutely affected turtles ($p<0.05$); however no significant difference was observed when analysis by grade was limited to turtles with thyroid spirorchiids only (negative animals not included). No significant relationships between infection or grade of infection and turtle size were detected.

Severity of associated pathological lesions correlated with numbers of distribution of eggs. In grade 3 infections, the thyroid gland often appeared black on section due to the numbers of spirorchiid eggs, which formed large egg masses within the thyroid and surrounding cervical vasculature. Histologically, these lesions were characterized by an intense mononuclear infiltrate, granulomatous inflammation, thrombosis, and loss of thyroid follicles. Adults and

vascular lesions, including medial hypertrophy and vasculitis, were observed in venules and arterioles.

Adult spirorchiids also were frequently observed in venules of the adrenal glands. Among category 1 and 2 turtles, adult *Neospirorchis* sp. were observed in 32.0% (16/50). No egg masses were observed in this location, but individual embolized spirorchiid eggs were common (not included in period prevalence). Adrenalitis was minimal or mild in all cases and consisted of mild, mononuclear perivasculitis and egg granulomas.

Other endocrine organs in which *Neospirorchis* species also were observed included the pineal gland (adults [n=7] and eggs), parathyroid gland (eggs only), pancreas (eggs only), and pituitary gland (eggs only). These observations were not included in any analyses due to small sample sizes.

***Neospirorchis* sp.: thymus.** Another common site in which spirorchiids were observed was the thymus, which was infected in 48.7% (37/76) of category 1, 2, and 3 turtles (Table 2-10). The most commonly observed gross finding was egg masses within thymic vessels (Figure 2-14). All but one turtle with intrathymic spirorchiid eggs and/or adults also had thyroid involvement. Of the thymic infections observed, 27.0% (10/37) were grade 3. When categorized by duration of illness, chronically ill turtles included a significantly higher proportion of thymic infections than acutely ill turtles ($p < 0.0001$). Significant differences in proportion of infection turtles were detected when turtles were categorized by size. More turtles in the ≤65 cm and >65-85 categories were infected than turtles >85 cm ($p < 0.05$). No significant relationships between grade of infection and duration of illness or size were detected.

***Neospirorchis* sp.: gastrointestinal tract.** The period prevalence data reported in this section include *Neospirorchis* species in which adults were found within the enterocolic

submucosa and produced large black, serpiginous egg masses (typically 2.0 to 5.0 cm in length) that were visible from the mucosal surface (Figure 2-15). The enterocolic *Neospirorchis* sp. were found in 62.0% (31/50) of the study population (Table 2-11). There were proportionately more infected chronically ill turtles than acutely ill animals ($p < 0.005$). No significant differences in grade were detectable between duration of illness categories. When categorized by size, there was a higher proportion of infected turtles in the >65-85 SCL size group than in the >85 cm size group ($p < 0.01$). No differences in grade of infection were observed among the different size groups.

Submucosal egg masses produced ulceration and secondary bacterial infection as eggs migrated through and injured the mucosa (Figure 2-16). Associated ulcerative enteritis was most severe in grade 3 infections in which large areas of the mucosal surface were affected. Lesions were most common in the small intestine, where egg mass numbers and density were the greatest.

There were observable differences in the morphology of adult *Neospirorchis* when specimens were examined under a dissecting microscope (Figure 2-17). Some individuals appeared longer, robust, and yellow as compared to others that were narrower in diameter and white. The eggs associated the more robust type also were larger (average of 59 x 47 µm) as compared to those of the more petite forms, which averaged 43 x 36 µm (Figure 2-17). In addition, both forms lacked the prominent pigmented cecae that were easily observed in adults recovered from the nervous system and endocrine organs (Figure 2-17).

Additional egg masses were observed during examination of the alimentary mucosa and urinary bladder mucosa using a dissecting microscope (Figure 2-18). These egg masses were smaller (<1.0 - 2.0 mm diameter) and lighter brown than the more commonly observed

enterocolic forms and the eggs were characterized by distinct fine surface projections (Figure 2-19). These small egg masses were found in ten *C. caretta* and locations included the stomach (n=1), M2 (n=2), M3 (n=2), cloaca (n=5), and urinary bladder (n=5). No adult spirorchiids were observed in association with these egg masses in any case.

***Neospirorchis* sp.: heart and major arteries.** The one identifiable species of *Neospirorchis*, *N. pricei*, was found in six *C. caretta*. When infected turtles were categorized by duration of illness, three were chronically ill and three were in the intermediate category. All infected turtles were >65 cm SCL, three were >65-85 cm SCL and three were >85 cm SCL. Most adults were removed from the heart in all but one case. Small numbers of adults also were collected from the mesenteric vessels (n=3), aortas (n=1), and carotid artery (n=1). It was not possible to confidently quantify the numbers of adults collected because individuals frequently were fragmented and often were extensively intertwined in the muscular ventricular trabeculae and were extremely difficult to flush out of the heart. Total numbers of complete and fragmented individuals from each case were 85, 24, 20, 9, 8, and 1. One turtle infected with *N. pricei* died of septicemia and thromboembolic disease and had dense aggregates of eggs within the atria over which mural thrombi had formed (Figure 2-20).

Other findings related to *Neospirorchis* sp. Adult *Neospirorchis* species were observed in several addition sites either by organ lavage, subgross examination, or histological examination. The observations were limited to a few individual turtles. The most extensive injury was observed in the testes of three *C. caretta* in which adults were present and abundant eggs were seen associated the granulomatous inflammation (Figure 2-21). One of these cases had a severe associated orchitis and moderate severity was observed in the other cases. Several specimens of *Neospirorchis* species were flushed from the hepatic vasculature of one *C. caretta*

and microscopic trematodes, consistent in morphology with *Neospirorchis* sp. were observed in the sinusoids of three additional turtles. No associated inflammatory response in the liver was observed. Additional locations were limited to single observations and rare or individual parasites. Sites included the lungs, nasal submucosa, arterial periadventitia, and bile duct periadventitia.

Spirorchiid impact rating

Spirorchiid infection in most of the turtles necropsied was categorized as either incidental (27/54) or undetermined (18/54), followed by contributory (6/54), uninfected (2/54), and fatal (1/54) (Table 2-12). Turtles were categorized by duration of illness and placed into two groups: an uninfected and incidental infection verses those turtles with undetermined, contributory, or fatal impact ratings. There were significantly more acutely ill turtles in the uninfected and incidental group as compared to the chronically ill turtles ($p<0.005$). No significant differences were detected when the same comparisons were made among size classes.

Criteria for categorization of spirorchiid significance in contributory, fatal, and undetermined categories reflect common arrays of findings. Within the contributory category, 5/6 turtles were emaciated and had multi-organ lesions. The key determining factor for assigning a spirorchiid impact rating of contributory, as stated in the scoring criteria, was the presence of significant multisystemic lesions associated with eggs or adult parasites. Of these cases, one turtle had extensive ulceration of the pylorus as a primary necropsy finding that was associated submucosal embolization of *Hapalotrema* eggs. Severe arteritis (*Hapalotrema mistroides*) was observed in four turtles, thyroiditis (*Neospirorchis* sp.) in two cases (one of which also had thymitis), and two cases had abundant (grade 3) meningeal spirorchiids (*Neospirorchis* sp.). Considerable embolization of eggs was interpreted as contributory in three cases in which abundant eggs were found in the gastroenteric submucosa and lungs. One case

also had abundant enteric *Neospirorchis* egg masses with associated enteritis and another had severe orchitis (*Neospirorchis* sp.). The only non-emaciated turtle in the contributory category was an underweight animal with septicemia and thromboembolic disease. In this case, atrial thrombi formed over areas of intense endocardial *Neospirorchis* egg deposition.

The only case in which spirorchiidiasis could be confidently confirmed as the cause of death was a loggerhead with massive egg granulomas in the gastrointestinal tract, liver, gall bladder, and other organs associated with a novel *Hapalotrema* sp. The extent of spirorchiid egg granulomas in this case resulted in secondary ischemia of the enteric mucosa, ulceration, and probable septicemia.

Of those cases in which the impact of spirorchiidiasis was undeterminable, half (8/18) were chronically ill and half (10/18) were in the acutely ill or intermediate duration categories. As stated in the scoring criteria, all had identifiable causes of death or multiple findings that could not be confidently associated with spirorchiidiasis based on lesion location and disease process or severity. For example, the chronically ill turtles were emaciated and had findings identifiable as a proximate cause of death or contributing health problems, but did not have the multisystemic lesions comparable to those given a contributory rating. The most common spirorchiid associated lesions in animals in the undeterminable category was grade 3 arteritis (12/18), neurospirorchiidiasis (6/18), and thyroiditis (6/18). Of these animals, 11/18 had lesions involving more than one organ or system, typically arteritis and neurospirchiidiasis or thyroiditis. Findings represented by individual cases included one turtle with large numbers of *N. pricei* in the heart, one animal with severe thymitis (concurrent with thyroiditis), and one case with large numbers of eggs embolized in the enteric mucosa.

Chelonia mydas

Study population demographics and duration of illness categories

Fifty-nine *C. mydas* were examined. The total number of *C. mydas* in each necropsy data category by region of stranding is shown in Figure 2-22. Frequency distribution of turtles by SCL is shown in Figure 2-23. The study population was heavily biased towards smaller, immature animals less than 45 cm SCL. The female to male ratio was 2:1 (38/19) (data unavailable for 3 turtles). Category 1 and 2 data included 50 turtles, 37 in the acute insult category, 8 in the intermediate duration category, and 5 in the chronic insult category (Table 2-13). The large number of turtles in the acute insult category reflected 30 animals that stranded during a cold-stunning event in St. Joseph Bay in January 2008. In addition, all turtles greater than 65cm SCL fell within the acute insult category, thus the intermediate duration and chronic insult categories were comprised completely of turtles less than 65 cm SCL. As in *C. caretta*, a primary reason for this bias was acute traumatic deaths of adults during breeding season.

Primary diagnosis or cause of death by duration of illness

Acute insult. Most of the *C. mydas* cases in the acute insult category were deaths associated with hypothermic stunning (cold-stunning) (30/37) and traumatic injuries (7/37). The hypothermia cases all originated from a single event in Zone 1 in January 2008. Most of the traumatic injuries (6/7) were watercraft-related. A seventh turtle was a nesting female that died from suffocation after a dune collapsed on her while nesting (1/6).

Insult of intermediate duration. Cases classified as insults of intermediate duration included turtles with traumatic injuries (3/8), infectious diseases/inflammatory conditions (3/8), and fibropapillomatosis (2/8). Two turtles with traumatic injuries had watercraft-related injuries with secondary bacterial infection (2/3) and one had a chronic, strangulating entanglement wound on its neck (1/3). Infectious diseases and inflammatory conditions included fungal

pneumonia (1/3), fungal tracheitis (1/3), and probable septicemia (1/3). Of the two turtles in which fibropapillomatosis was the primary finding, one had been euthanized due to the presence of visceral tumors, and one had a severe marine leech infestation (*Ozobranchus* sp.) and anemia resulting in secondary drowning after entering a coastal power plant.

Chronic insults. Health problems observed in chronically ill *C. mydas* were varied. Primary diagnoses included bacterial meningoencephalitis (1/5), fibropapillomatosis (1/5), and a chronic entanglement wound (1/5). Emaciation was the only significant finding in one animal (1/5) and the death of another was interpreted as multifactorial (1/5).

Prevalence by genus and identifiable species (category 1 & 2 data).

Three genera of spirorchiid trematodes were detected in the study population, including *Neospirorchis*, *Learedius*, and *Hapalotrema*. Period prevalence of *Neospirorchis* was 92.0% (46/50), *Learedius* was 8.0% (4/50), and *Hapalotrema* was 2.0% (1/50). All *Learedius* specimens were identified as *L. learedi* and the only example of a *Hapalotrema* infection was identified as *H. postorchis*. Type I eggs (*Hapalotrema* sp. or *Learedius* sp.) were observed in two additional turtles from which no adults were recovered. Among infected *C. mydas*, the majority 45/50 were infected by one genus and five were infected by two different genera.

As in *C. caretta*, specific species identification of *Neospirorchis* specimens was not possible due to the inability to obtain intact parasites suitable for identification. Classification of adult and egg specimens as *Neospirorchis* was based on egg morphology and elongate body type.

Site predilection and associated pathological lesions

Learedius learedi. Sites from which *L. learedi* were recovered included the heart and hepatic vessels. Three of the four infected turtles were adults and one was an immature animal with a SCL of 25.6 cm. Thus, out of 46 immature turtles examined (category 1 & 2 data), only 1

had evidence of *L. learedi* infection. The three adult turtles died from traumatic injuries (acute insult category) and the juvenile was underweight and had fungal pneumonia (intermediate duration category). The latter animal is estimated to have had at most 1-2 adults, which were observed on histological examination and did not lavage from the heart during parasite recovery. The adult turtles had intensities of 21, 35, and 36 adult parasites per host. Two of these turtles had associated distinct focally extensive endocarditis located supravalvular to the atrioventricular valves (Figure 2-24). These lesions were observed grossly as well-demarcated, raised, tan pink areas with an irregular surface and histologically were characterized by a mixed mononuclear and granulocytic infiltrate, fibrosis, and surface villous proliferation. Milder, more diffuse endocarditis also was present in both turtles. In addition, both animals with endocardial lesions also had severe thickening of the aortic system due to marked subintimal proliferation and mild pleocellular endarteritis. The extent of these lesions in one case was more extensive and involved the pulmonary, brachiocephalic, carotid, mesenteric, and associated smaller arteries, as well as prominent generalized thickening/hypertrophy of the myocardium (Figure 2-25).

Hapalotrema postorchis. Only one *C. mydas* was confirmed to be infected by a species of *Hapalotrema*, which was identified as *H. postorchis*. This parasite has not been previously reported in Florida. The infected turtle was an adult male that died as the result of head trauma sustained during entrainment in a coastal power facility (acute insult category). Twenty-one adult *H. postorchis* were collected and fragments of an additional four to ten individuals were observed. The aortic system was prominently thickened, which reflected chronic arteritis similar to that observed in turtles infected with *L. learedi* (Figure 2-26). The left aorta was most severely affected and had multifocal, grossly visible plaques of endarteritis containing several

embedded *H. postorchis*. Lesions in small arteries, including the mesenteric, renal, adrenal, and carotid arteries, also were observed.

***Neospirorchis* sp.** As in *C. caretta*, spirorchiid eggs consistent in morphology with *Neospirorchis* sp. were observed embolized throughout the body and were most readily visible in the lung and submucosa of the gastrointestinal tract. In addition, dense concentrations of eggs and/or adult parasites were found in some locations similar to that observed in *C. caretta*, specifically the central nervous system, endocrine organs, and rarely within the gastric submucosa. The period prevalence of *Neospirorchis* species found in these sites for the four necropsy categories is shown in Table 2-14.

***Neospirorchis* sp.: central nervous system.** Adult spirorchiids and/or egg masses identified as *Neospirorchis* sp. were found in leptomeninges of 11.9% of necropsied *C. mydas* (7/59). These findings in *C. mydas* must be qualified with regard to grade and size class considerations. Foremost, all egg masses tended to be very small (often 1 mm or less), thus the criteria used to quantify/grade *C. caretta* infections were scaled by abundance of eggs, rather than egg mass size to represent equivalent abundance in *C. mydas*. Second, adult parasites only were observed in subadult or mature *C. mydas*, which included 5/7 infected turtles. Four were adults with SCL of 95 cm or greater that died from acute traumatic events during nesting season. The fifth turtle was submitted as a head only and based on its size was estimated to be a large subadult or adult. Thus, all of the mature greens examined had neurological infections. Two cases were classified as grade 1 and three as grade 2. The remaining two turtles were immature, one of unknown size and another with a SCL of 43.1 cm. In both cases, no adults were observed and egg masses consisted of very small numbers of eggs (both grade 1), which may reflect embolization rather than oviposition within meningeal vessels, as is apparent when adult

parasites are present. Nevertheless, these cases were included because similar patterns of egg embolization (from distant sites) were not observed in the other 52 turtles examined.

***Neospirorchis* sp.: endocrine organs.** Spirorchiid parasitism of the thyroid gland was observed in 33.3% (17/51) of necropsied *C. mydas* (Table 2-14). Of the infected turtles, adult parasites were observed in 58.8% (10/17) and 5.9% (3/51) were classified as grade 3 infections (Figure 2-27). Comparisons with other factors were limited given the biases of the study population, and limited number of chronically ill, mature, and large immature turtles examined. Associated pathological lesions were as described for *C. caretta*.

Adult parasites were recovered from the pineal gland in two turtles and eggs were focally concentrated at this site in a third case. One turtle was found to have adult *Neospirorchis* sp. in the parathyroid gland. Findings in other endocrine organs were limited to embolization of eggs. No adult parasites were observed in the adrenal glands.

***Neospirorchis* sp.: gastrointestinal tract.** The only spirorchiid-related necropsy finding in the alimentary tract that exhibited a distinct pattern, other than widespread or regionally intense egg embolization, was the finding of small egg masses within the gastric submucosa (Figure 2-28). Egg deposition at this location was observed in 10.4% (5/48) of examined *C. mydas*, which were all immature and represented all duration of illness categories. This finding typically only was visible by examination of the gastric mucosa under a dissecting microscope and associated inflammatory lesions were minimal or mild. No adult spirorchiids were observed grossly, but were seen histologically in two cases. In an additional case, similar eggs and a single adult spirorchiid were observed in the small intestine by histological examination. Other than this example, no discrete enteric *Neospirorchis* egg masses, as observed in *C. caretta*, were seen in *C. mydas*.

Other findings related to *Neospirorchis* sp. In addition to finding adult spirorchiids and egg masses in the above anatomical locations, widespread egg embolization in relatively high densities were observed in seven cases, all but one of which were immature turtles in the acute insult category. Eggs were most readily observed in the lungs and gastrointestinal mucosa of four cases, primarily the lungs in two cases, and primarily the gastrointestinal tract in one case (Figure 2-29. Adult parasites and/or egg masses in these cases was limited to the thyroid gland, which was parasitized in four turtles.

Spirorchiid impact rating

Most spirorchiid infections in *C. mydas* were in the incidental category (35/50), followed by undetermined (10/50), uninfected (4/50), and contributory (1/50) (Table 2-16). Spirorchiid infection was not identified as the cause of death in any *C. mydas*. The size class and duration of illness bias in the study population prevented comparison of rating within these categories.

Three findings of concern were observed in *C. mydas* with infections that were categorized as undeterminable impact: cardiovascular disease associated with *L. learedi* and *H. postorchis* in adult turtles (n=3), severe (grade 3) parasitism of the thyroid gland in immature turtles (*Neospirorchis* sp.) (n=3), and intense egg embolization in the lungs and/or gastrointestinal tract of immature turtles (*Neospirorchis* sp.) (n=4). Three of the four latter cases also had grade 2 thyroid parasitism. The three adult turtles were all males that died of acute traumatic injuries. Six of the immature turtles died from hypothermic stunning. The tenth case was an emaciated immature turtle with severe fibropapillomatosis and presumptive septicemia.

The single case in which spirorchiidiasis was interpreted as contributory to death had massive numbers of *Neospirorchis* sp. within the pulmonary vasculature and severe fibropapillomatosis. The lungs in this case were discolored due to the large numbers of spirorchiid eggs.

Body condition indices in hypothermic-stunned immature *C. mydas*

Body condition indices were calculated for the thirty *C. mydas* that died of hypothermic stunning (Table 2-17). One case was discarded as an outlier because abnormal conformation of the carapace (old trauma) affected BCI calculations. No significant differences were observed in body condition indices, using either calculation, when turtles were compared based on the presence/absence of *Neospirorchis* in the thyroid gland or presence of relatively large numbers of *Neospirorchis* eggs concentrated in one or more organ systems. The latter group primarily included turtles with large numbers of intra-thyroidal eggs and moderate to large numbers embolized to the lungs and alimentary submucosa.

Discussion

The study populations of *C. caretta* and *C. mydas* reflect the predominant size classes of each species represented in the historical stranding data for Florida (Foley et al., 2007). There are, however, factors that must be considered in any extrapolation of findings of this study to the general stranding population. The goal of necropsy efforts was not to mimic actual demographic proportions of the total stranding population, but to include representation of major size classes to gain a more complete understanding of the host-parasite relationship. With the exception of a few studies, correlation of spirorchiid data with size classes frequently has been overlooked yet is critical to understanding host-parasite relationships in species with a complex life history and broad geographical range. In addition, turtles with acute insults were sought whenever possible to avoid the bias and confounding health issues that limit interpretation of findings in chronically ill animals, which comprise a large proportion of stranded sea turtles. Thus, the design and execution of this study tempered examination of a representative subset of animals with pursuit of fundamental questions regarding spirorchiidiasis in Florida sea turtles.

The study population included 148 stranded turtles, 89 *C. caretta* and 59 *C. mydas*. Included in the category 1 and 2 data were 57 turtles identified that died of relatively acute insults, the most common examples of which included hypothermic stunning (*C. mydas*, n=30), traumatic injury (both species, n=13), brevetoxicosis (*C. caretta*, n=7), and an unsolved MME (*C. caretta*, n=4). Twenty-two turtles of both species died from illnesses classified as intermediate duration, which included animals that stranded in thin nutritional condition during the unsolved MME (*C. caretta*, n=6), chronic traumatic injuries (both species, n=5), miscellaneous infectious diseases (both species, n=8), fibropapillomatosis (*C. mydas*, n=2), and enteric impaction (*C. caretta*, n=1). The chronic insult category included twenty-five turtles, the deaths of more than half of which were classified as multifactorial (n=14) and reflected a combination of advanced poor nutritional condition and opportunistic disease processes.

Three major features of the study population and categorization of data require discussion with regard to rationale and implications on data interpretation. First, most *C. mydas* died during single hypothermic stunning event, which provided 30/59 *C. mydas* examined and 30/37 of those classified with acute insults. Although inclusion of these turtles biased the total study population, the event provided an opportunity to examine a relatively large number of seemingly robust *C. mydas* with a known cause of death and was insightful in interpreting the significance of spirorchiidiasis. However, as outlined in the following discussion, this bias must be considered when comparing these data to other size classes, as well as turtles from other regions. Second, the use of an intermediate duration of insult category arguably was a very subjective classification because it included turtles ranging from mildly underweight to visibly thin, but not in sufficiently poor condition to be classified as emaciated, chronically ill turtles. The necropsy and parasitological data, however, support this categorization. Most turtles included in the

intermediate duration category had singular identifiable causes of death, as did the acute insult category, in contrast to those turtles with advanced chronic disease. Furthermore, in the various aspects of spirorchiidiasis examined, data for turtles in the intermediate duration category often fell between statistically significant differences observed between the acute and chronic categories, as expected in a continuum of disease relationships. Lastly, the bias produced by proportionately more adult *C. caretta* in the acute insult category may have confounded some of the statistical analyses, specifically the differences noted among size classes of turtles with thymic and enteric spirorchiidiasis. In both instances, proportionately more turtles in the smaller size categories were infected as compared to adult turtles; however, there also were more chronically ill turtles infected as compared to animals that died of acute problems. Thus, the differences noted in the size class of infected turtles actually may have reflected the greater proportion of smaller turtles in the chronically ill group.

Assessment of the significance of spirorchiidiasis in the study population was one of the primary objectives of this study. The spirorchiid impact rating was devised as conservative means for estimating significance and distinguishing pathological lesions of concern (but of unknown significance) from those in which a causal role in death and stranding could be reasonably inferred based on complete necropsy. In *C. caretta*, spirorchiidiasis was interpreted as contributory to death or causal in 13.0% (7/54) of examined animals, and was a finding of concern, but of unknown significance, in an additional 33.3% (18/54). Of the latter group, it is notable that over half (10/18) of those animals were in the acute or intermediate illness categories. Thus, relatively robust turtles with unrelated causes of death had pathological lesions of concern based on severity. In *C. mydas*, spirorchiidiasis was found to be contributory to death in only one case (2.7%, 1/37) and no examples of fatal spirorchiidiasis were observed. The

proportion of *C. mydas* in the undermined category, however, was comparable to *C. caretta* and most (9/10) *C. mydas* in this category died of acute insults. These findings warrant comparison with the only published large study of spirorchiid-associated pathological lesions in sea turtles, which examined *C. mydas* stranded in Moreton Bay, Queensland, Australia (Gordon et al., 1998). Several notable similarities and differences between this study and the current study will be cited throughout this discussion. Gordon et al. reported spirorchiidiasis as the cause of death or a contributory cause in 10.4% (10/96) and 30.2% (29/96) of stranded turtles, respectively, which, at first glance, appears much greater than was observed in Florida turtles, especially *C. mydas*. The disparity in these findings may be due to demographical and geographical differences in the study populations, the criteria for interpreting significance, and health status at the time of stranding. Unfortunately, the size classes of examined animals were not given in the Australian study, which omits a critical component of the host-parasite-disease relationship. In addition, the criteria for determining spirorchiidiasis as the cause of death were not stated, which also severely limits comparisons among studies. The authors certainly describe significant pathological lesions in select cases, such as occlusive aortic thrombi, septic thrombosis, and intracranial hemorrhage; however, the precedence for vaguely concluding cause of death relationships in the spirorchiid disease literature prevents confident comparison of interpretative opinion. The other significant difference between the Moreton Bay *C. mydas* study and the present study is that many of the Australian turtles appear to have been chronically ill, whereas a significant number (71/106) of Florida turtles died of acute insults. If examination had been limited to chronically ill *C. caretta* (*C. mydas* were underrepresented), the proportion of causal (5.0%, 1/20) and contributory (25.0%, 5/20) relationships actually are relatively comparable to the Australian *C. mydas* study. In recognition of the caveats considered when examining

chronically ill animals, Gordon et al. (1998) stated that further examination of turtles with acute illness is necessary to better define incidental or "harmless," i.e. background, infections and lesions.

Another previous study that bears comparison is a relatively recent investigation of epizootiology of spirorchiidiasis in Hawaiian *C. mydas* that examined splenic egg counts (Work et al., 2005). This study found that splenic egg density was inversely related to both SCL and BCI. Regional relationships also were identified. These trends contrast with some of the findings of the present study in that some of the parameters measured, e.g. neurospirorchidiasis, were greater in larger turtles. There are several factors that must be considered in comparing these studies. First, as noted by investigators in the Hawaiian study, splenic egg density likely reflects chronic accumulation and may not correlate with intensity of adult parasite infection, whereas the Florida study documented active infections with lesions associated with adult spirorchiids. Second, there appear to be differences between the spirorchiid fauna of Hawaiian *C. mydas* and Florida turtles, the most significant of which is the apparent absence of *Neospirorchis* species in Hawaii. *Neospirorchis* was the most commonly detected genus in both *C. caretta* and *C. mydas* in Florida and lesions associated with members of this genus were a major finding in the present study. Splenic egg density may not accurately reflect the selective accumulation of *Neospirorchis* eggs in various anatomic sites. Minimally, this technique would be difficult to validate for use in Florida turtles given the spectra of lesions and sites parasitized by *Neospirorchis* in both *C. caretta* and *C. mydas*.

Several pathological lesions of concern associated with spirorchiids were documented in Florida turtles. These observations provided further information on recognized problems, such as neurospirorchiidiasis, and identified new or poorly documented health issues, such as major

arterial disease and spirorchiid-associated thyroiditis. It is not prudent to disregard these findings as insignificant or merely incidental, despite the fact that many examples were documented in turtles that died of unrelated acute problems. Rather the lesion severity and potential for physiological effects or sequelae suggest a negative impact on host fitness. Furthermore, infection and severe associated lesions in relatively healthy turtles support that spirorchiids can act as primary pathogens, i.e. they have the ability to produce injury in otherwise healthy individuals, as opposed to a more limited role as secondary, opportunistic agents in debilitated animals. Conversely, these otherwise robust and, in many cases, actively foraging infected turtles with pathological lesions provide an important context in which to interpret similar findings in chronically ill animals with other confounding health issues. Thus, based on the findings of this study, spirorchiids are regarded as potential primary pathogens and a health concern in free-ranging turtles, but interpretation of significance in individual cases requires caution, especially with regard to concluding proximate cause of death.

The high prevalence of major arterial disease primarily associated with *H. mistroides* in *C. caretta* was notable and underscores the importance of complete examination of the aortic system and larger arteries when performing necropsies. There were no significant differences among duration of insult categories, and severe lesions were observed in turtles that died of acute traumatic injuries and brevetoxicosis. Significantly more adult turtles were infected by *Hapalotrema* sp. and more had associated arteritis, which suggests that age-related factors, such as exposure over time or differences in exposure intermediate host(s), are present. Furthermore, the occurrence and population significance of *Hapalotrema*-associated arteritis may be underestimated as adult *C. caretta* comprise a small proportion of the stranding population.

Although different spirorchiid species were involved, a similar picture emerged in *C. mydas* of this study. Three of four adult *C. mydas* examined had severe arterial disease associated with *L. learedi* and *H. postorchis*. In contrast, infection by *L. learedi* was only observed in one of 46 examined immature *C. mydas*, and very few parasites and no significant lesions were present in this single case. Thus, from this sampling, *Learedius* and *Hapalotrema* appear to be more of a health issue in adult *C. mydas* in Florida waters. This observation is consistent with a parasitological survey of nesting females in Tortuguero, Costa Rica, in which *L. learedi* was found in 39 of 40 turtles (Santoro et al., 2006).

There were some notable differences in the arterial lesions observed in the present study and those described by Gordon et al. (1998). Both studies observed lesions in the heart and major arteries in significant numbers of stranded turtles. Although different spirorchiid species were identified, the left aorta was the most common site of both lesions and localization of adult spirorchiids. Gordon et al. described arterial thrombus formation, mural and occlusive, in 81 turtles, as well as an interesting pattern of "externalization" of thrombi. In contrast, in category 1 necropsies, mural thrombus formation was only observed in nearly half of affected turtles (10/24) and only one of these had a partially occluding luminal thrombus. Part of the discrepancy may lie in use of the term thrombus. Gordon et al. include descriptions of spirorchiid eggs and pigmented debris, some of which appeared to be involved in the exteriorization process, in their definition of thrombi. In the current study, the use of the term thrombus was limited to lesions characterized by the formation of fibrin clots on the endothelial surface or within the vessel lumen. The exteriorization process, as described by Gordon et al., was not observed, although the inflammation was transmural in 15 or 24 cases examined histologically and there were granulomas containing spirorchiid eggs or pigmented debris

observed within these lesions. In a few cases, the impression of an exteriorization process could be claimed. In addition to semantics, some of the variation in desribed lesions could reflect differences in lesions associated with different species of spirorchiids. Specific identification only was performed for a small subset of turtles in the Australian study, and identified species in the heart and major vessels included *H. pambanensis* and *H. postorchis*. There was only a single example of arteritis associated with *H. postorchis* infection in the current study population and severe lesions were more often associated with *H. mistroides* infection in *C. caretta*.

Although the large pigmented thrombi as described by Gordon et al. (1998) were not observed, an interesting pattern of degeneration and necrosis of the tunica media was seen in 7 of 24 category 1 cases. It was remarkable that sequelae, such as thrombosis, aneurysm formation, or rupture were not observed more frequently given the severity of some of these lesions. This pattern of medial necrosis has not been previously described in spirorchiid-associated lesions.

Multiple spirorchiid species, including *N. pricei*, *H. pambanensis*, *H. mistroides*, and *L. learedi* were recovered from the hearts of Florida turtles. Patchy areas of mural endocarditis were observed in many of these cases, but distinctive lesions also were noted in turtles infected with *N. pricei* and *L. learedi*. One turtle infected with *N. pricei* had intense areas of egg accumulation within the atria with associated atrial thrombi and thrombolic disease resulting from septicemia. Intense egg accumulation in the atria was limited to this one of six turtles infected with *N. pricei*. Only one *C. mydas* was found to have an adult spirorchiid in the heart that was consistent in morphology with a *Neospirorchis* species. Presumptive identification was based on histological examination because apparent very few (possibly one) adult parasites were present and did not flush from the ventricle during parasite collection procedures. Two *Neospirorchis* species have been previously reported from hearts of sea turtles, *N. pricei* from *C.*

caretta and *N. schistosomatoides* from *C. mydas*. Another example of a distinct cardiac lesion was observed in two *C. mydas* infected with *L. learedi*. Both turtles had well-demarcated areas of papillary endocarditis associated with attached *L. learedi* located supravalvular to the atrioventricular valves. Similar lesions have been observed in captive turtles at the Cayman Turtle Farm, Limited associated with *L. learedi* infection, as well as in nesting females from Tortugeuro National Park, Costa Rica (unpublished data). Also, Gordon et al. (1998) describe a highly similar lesion at this location in Australian *C. mydas*, but did not report *L. learedi* as one of the identified spirorchiid species. Perhaps infection by *L. learedi* was overlooked in the 1998 study (only a small subset of parasites were identified) or the supravalvular location is a common attachment site for both *Learedius* and *Hapalotrema* species.

Neurospirorchiidiasis (*Neospirorchis* sp.) has been a concern in *C. caretta* in Florida since being implicated in a mass mortality event in south Florida in 2000 and 2001 (Jacobson et al., 2006). The findings of this study have identified several features of neurological spirorchiid infection in *C. caretta* that are critical to future disease studies and better understanding of the host-parasite relationship. First, neurospirorchiidiasis was observed in nearly half of the study population (44/89), of which 64.4% (29/45) were classified as incidental infections. Over 11.1% (5/45) of cases, however, were intense infections classified as grade 3. The major question regarding these parasites is their clinical or health significance. There are anecdotal reports of improvement of vague neurological symptoms following antihelminthic therapy; however, correlative clinical studies with confirmation of infection are lacking, primarily due to lack of methods for specific antemortem diagnosis. Unfortunately, the findings of this study do not answer these concerns. Of the five turtles with severe (grade 3) neurospirorchiidiasis, all either were found dead or died soon after discovery and did not receive a neurological examination.

Three of these animals were severely emaciated with multiple disease processes, including spirorchiid-associated lesions in other organs. Of the remaining two cases, one died of brevetoxicosis, and thus would have exhibited confounding neurological symptoms, and the other died of a gastrointestinal perforation and secondary coelomitis. Therefore, of these five cases, it can only be stated for a single turtle that abundant meningeal spirorchiids were observed, but were unrelated to cause of death based on good nutritional condition and known cause of death (gastrointestinal perforation). Of the 44 brains examined with neurospirorchiidiasis, no associated hemorrhagic or ischemic events were identified.

Another notable finding was the significant relationship between neurospirorchiidiasis and adult *C. caretta*. As with *Hapalotrema* infections, this observation may reflect increasing probability of exposure over time, selective exposure, or other age-related factors. An age-dependent effect is relevant to previous investigation of the 2000 and 2001 *C. caretta* mortality event in south Florida. At the time of the summarization and analysis of this event, estimated intensity of neurospirorchiidiasis was compared with existing unrelated cases in lieu of actual prevalence data. The "control" cases primarily were stranded turtles from northern Florida with a median SCL of 61.5 cm. In retrospect, this comparison was inappropriate given the higher prevalence of neurospirorchiidiasis in large turtles. Thus, any association of neurospirorchiidiasis and the 2000/2001 event cannot be concluded based on this comparison.

Neurological *Neospirorchis* infections were not limited to *C. caretta*, but also were observed in *C. mydas*. Similar to *C. caretta*, there appears to be a size relationship in stranded Florida *C. mydas* as well, as all of the infected turtles with adult parasites were adult or large subadult turtles. Neurospirorchiidiasis was not found in any of the smaller turtles, with only two possible exceptions in which eggs, but no adult parasites were observed. No infected *C. mydas*

had high intensity neurological infections. Neurospirorchiidiasis has been previously documented or depicted in photographs of *C. mydas* in at least three previous studies, although the genus was correctly identified in only one report (Glazebrook et al., 1989; Gordon et al., 1998; Raidal et al., 1998). Gordon et al. described microscopic flukes, consistent with *Neospirorchis* sp., in the meninges of 73.6% (53/72) of examined *C. mydas* (1998). The species *N. schistosomatoides* was identified in two of these cases.

Another pathological finding of concern in both species was parasitism of the thyroid gland, which resulted in extensive glandular injury in severe cases. The thyroid gland was one of the most common sites inhabited by spirorchiids in both *C. caretta* and *C. mydas*. Comparable proportions of the different age/size classes were infected. There were significantly more infections and more intense infections in chronically ill *C. caretta*. The same comparisons were not possible for *C. mydas*; however, two cases of grade 3 lesions were observed in relatively robust *C. mydas* that died of hypothermic stunning. Futhermore, there was no evidence of any significant effect on BCI in the immature *C. mydas* that died of hypothermic stunning that were observed to have intra-thyroidal *Neospirorchis*. The significance of thyroid injury associated with *Neospirorchis* infection requires further study.

There were several interesting differences in parasitism of endocrine organs by *Neospirorchis* sp. in the *C. caretta* and *C. mydas*. Infection of the thyroid gland was identified in both species; however, concurrent parasitism and egg embolism within the thymus, which was frequently observed in *C. caretta*, was not seen in *C. mydas*, nor has involvement of this site been described in the literature. Another notable difference was occurrence of adult *Neospirorchis* in the vasculature of the adrenal glands, which was observed in 32.0% (16/50) of category 1 and 2 *C. caretta*. Although “microscopic” flukes, presumably *Neospirorchis* sp.,

occasionally were observed in adrenal glands of *C. mydas* in a previous study, none were seen in the 50 examined Florida *C. mydas* (Gordon et al., 1998). These differences could reflect differences in spirorchiid species involved or variation in host-parasite interaction.

The organ system most commonly involved in spirorchiidiasis in terms of egg embolization and occurrence of adult spirorchiids was the gastrointestinal tract. Again, differences were observed between *C. caretta* and *C. mydas*. Adult *Neospirorchis* sp. within the enteric submucosa and localized egg deposition characterized by large black, serpiginous egg masses were observed in over 65.8% (50/76) of stranded *C. caretta*. Proportionately more chronically ill turtles were infected than turtles in the acute insult category, and all severe (grade 3) infections were in chronically ill animals. In contrast to spirorchiid infections in other organ systems, proportionately more infected turtles with enteric involvement were large immature turtles (>65-85 cm SCL group), as compared to mature turtles, and most of the grade 2 and higher infections were animals in this size class. This possible relationship with size class must be cautiously interpreted given the aforementioned bias in the study population. Wolke et al described bacterial enteritis associated with spirorchiid eggs, as was observed in the present study, in stranded Atlantic *C. caretta*, but did not identify the spirorchiid type. In addition, similar serpiginous *Neospirorchis* egg masses in the enterocolic submucosa were not observed in Florida *C. mydas* in the present study, although very similar egg masses have been described in *C. mydas* in Australia (Gordon et al., 1998).

Smaller submucosal *Neospirorchis* egg masses, visibly distinct from the larger serpiginous masses or diffuse embolization, were observed in the stomach of over 10.4% (5/48) of examined *C. mydas*, and were associated with adult parasites in histological sections in some cases. A

sixth *C. mydas* had similar egg masses in the intestine. These eggs were observed to have fine surface projections as was seen in eggs observed in the colon, cloaca, and stomach of *C. caretta*.

The other gastrointestinal lesions observed were those associated with *Hapalotrema* species in *C. caretta*, which ranged from incidental diffusely embolized eggs and embolized egg masses within subserosal and submucosal vessels to more substantial, transmural egg masses with associated ischemic lesions and ulceration of the stomach and intestine. One of these cases was the only confirmed example of fatal spirorchiidiasis. There may be differences in the enteric lesions associated with different *Hapalotrema* species. The egg masses in the fatal case, which was infected with a novel species of *Hapalotrema*, were much more extensive than was observed in turtles infected with *H. mistroides* and *H. pambanensis*. A second case with similar pathological lesions was observed in a *C. caretta* infected the novel *Hapalotrema* species, but was excluded from the study due to a partial data set. Additional lesions in the digestive tract associated with *Hapalotrema* eggs included enteric polyp formation, similar to schistosomal polyposis of humans (Mesquita et al., 2003), and papillary lesions in the gall bladder secondary to granulomatous cholecystitis.

The testes were another organ in which significant pathological lesions were associated with parasitism by *Neospirorchis* species. Although only observed in three turtles, these cases comprised 25.0% (3/12) of examined adult male *C. caretta*. There may have been persistent, negative effects on reproduction had these turtles recovered or been rehabilitated. This finding was not observed in *C. mydas* nor was parasitism of the ovary or other aspects of the female reproductive system identified in either species.

In addition to pathological lesions associated with adult parasites and/or concentrated egg deposition in specific sites, diffuse embolization of eggs, especially to the lungs and

gastrointestinal tract, was severe in a number of cases. With the exception of the aforementioned specific examples, most of the eggs were *Neospirorchis* species in both *C. caretta* and *C. mydas*. Multi-organ accumulation of eggs was a primary finding in 4/7 cases in which spirorchiidiasis was interpreted as contributory to the cause of death and was a lesion of concern in several turtles in the undeterminable significance category. Interpretation of significance must be approached very cautiously, as noted in the group of *C. mydas* that died from hypothermic stunning. Six of these turtles were observed to have moderate or large numbers of *Neospirorchis* eggs embolized to the lungs and/or gastrointestinal tract, most of which also had adult *Neospirorchis* and eggs in the thyroid gland. The significance of egg accumulation in these cases is unknown, which was reflected in the undeterminable impact classification. There was no observable effect on nutritional condition as assessed by body weight, abundance of internal fat, and body condition indices. Effects may manifest, however, when other confounding health problems, such as concurrent disease processes or environmental stressors, are present.

Spirorchiid infection (all species included) was observed in 96.4% (54/56) of *C. caretta* and 92.0% (46/50) of *C. mydas* examined in category 1 and 2 necropsies. These numbers were comparable to previous studies that also used targeted methodology to detect spirorchiid infection (Work et al., 2005; Santuro et al., 2006; Gordon et al., 1996; Dailey et al., 1992). Infection by multiple genera was frequently observed in *C. caretta* and all adult *C. mydas*, but rarely in immature *C. mydas*. The diversity of spirorchiid species identified in this study increase the number of species documented in the West Atlantic region. Species reported in the reviewed literature prior to 2004, included *C. bipora*, *H. synorchis*, *N. pricei*, and *N schistosomatoides*. The current study adds to this list *H. mistroides* and a novel *Hapalotrema* in *C. caretta*, and *H. postorchis* and *L. learedi* in *C. mydas*. The only spirorchiid species that was

previously documented in Florida waters that was not observed in the study population is *H. synorchis*. In addition to these species, there is potential for great unrecognized diversity in the genus *Neospirorchis* that could not be investigated by morphological examination due to the inability to collect intact specimens. There were obvious site predilections for adult *Neospirorchis*, including the leptomeninges, heart and major vessels, endocrine organs, thymus, and submucosa of the alimentary tract. Although intact adults could not be examined, there were distinct differences in size and general morphological features among adult parasites and eggs from different anatomic locations that require further study.

Notably, identifiable spirorchiid species were limited to either *C. caretta* or *C. mydas*. These records contribute to existing data on host range. Several marine spirorchiids are known to parasitize multiple sea turtles species and it has been generally stated that there is no evidence of any host specificity at the generic or species levels (Smith, 1972; Smith, 1997a). At least one study, however, has offered evidence of potential host restriction in freshwater spirorchiids (Byrd, 1939, cited in Goodman, 1987). It must be considered that any apparent host restriction in published reports could reflect bias as most studies have only examined *C. mydas*. Only few studies have examined both *C. caretta* and *C. mydas* inhabiting the same general region and all examined either an unspecified number of or very few turtles (Simha and Chattopadhyaya, 1980; Platt and Blair, 1998; Loss, 1902). Thus far, *L. learedi* has only been described in *C. mydas* and *E. imbricata*, and reports of *H. postorchis* are limited to *C. mydas* (Smith, 1972; Smith, 1997b). Neither of these species was observed in Florida *C. caretta* included in the current study, thus infection may be rare or absent in this species, at least in this region. Another *Hapalotrema* species, *H. pambanensis*, has only been reported in *C. mydas* and *E. imbricata* (Smith, 1997b). Based on the current study, *C. caretta* is now added to definitive hosts for this species. None of

the specimens of *H. mistroides* or the novel *Hapalotrema* were collected from *C. mydas*; however, examination of additional larger turtles is necessary given the apparent rarity of *Hapalotrema* species in smaller immature *C. mydas* in Florida waters. Lastly, the single species of *Carettacola*, *C. bipora*, was limited to *C. caretta*, which was the identified host in the only report and original description of this species (Manter and Larson, 1950). Evidence of specialization or host restriction of marine spirorchiids requires further investigation. Results from the present study of Florida turtles support that there are at least large differences in regional prevalence among *C. caretta* and *C. mydas*. Differences in parasite fauna would not be surprising given the differences in diet and habitat utilization between the two turtle species.

The occurrence of adult parasites and egg deposition/accumulation in specific sites may reflect some degree of microhabitat selectivity. There are reports of both host-organ specificity, as well as lack of specific tropisms in the freshwater spirorchiid literature (Wall, 1951; Holliman et al., 1971; Platt, 1993). It is reasonable to hypothesize that specialization would evolve, in at least some circumstances, as it has in mammalian and avian schistosomes (Marquardt et al., 2000). Although this issue will require additional techniques, e.g. molecular characterization, to further study *Neospirorchis* species, there is evidence of site tropism in the more easily studied linguiform genera. All reports of *Carettacola* species in which anatomic location is specified have collected adult parasites from hepatic blood vessels (Dailey et al., 1991; Dailey et al., 1992; Graczyk et al., 1995). Similarly, all adult *C. bipora* in the present study were collected from either hepatic vessels or mesenteric vessels, the latter of which may have reflected presence of parasites in extrahepatic portal circulation. Similarly, the three *Hapalotrema* species identified were observed primarily in the heart and major arteries in this study and in previous reports (Smith, 1997b). Both *H. mistroides* and *L. learedi* also have been reported in hepatic circulation

(Smith, 1997b; Santoro et al., 2006), as observed in the present study. Although adult spirorchiids have been collected from these various vessels, there appears to be a predilection for the left aorta in some *Hapalotrema* species, as observed by Gordon et al. (1998) and for *H. mistroides* and *H. postorchis* in Florida turtles. The left aorta is the major conduit to the superior mesenteric artery, and thus provides access to smaller enteric vessels where eggs may migrate to the mucosa and reach the external environment.

Microhabitat utilization is relevant to both health effects in the turtle host and life cycle of the parasite. Sites where eggs accumulate or where adult parasites, e.g. *Hapalotrema* species, cause inflammation and tissue injury may affect organ function or result in pathologic sequelae, such as enteric ulceration or arterial thrombosis. In addition, in sites where external access is seemingly remote, such as the brain or thyroid gland, the strategy for propagation of the life cycle is unclear. Similar observations have been made in freshwater turtles. Holliman (1971) suggested that the death of hyperinfected turtles may contribute to the parasite life cycle by releasing eggs entrapped within internal tissues. In dead stranded sea turtles examined in the present study, live miracidia were observed in spirorchiid eggs following postmortem intervals of 48 to 72 hours, which is ample time for dispersal of a carcass by marine scavengers. Another scenario that has been anecdotally discussed is the possibility that a given sea turtle species is an aberrant host in some instances, such as in cases of neurospirorchiidiasis in *C. caretta*. However, the high prevalence and apparent incidental nature of most infections, as observed in this study, would be an unusual presentation of an aberrant host. Alternatively, the numbers of eggs that reach the lungs and/or gastrointestinal tract over time may be sufficient for shedding.

Conclusions

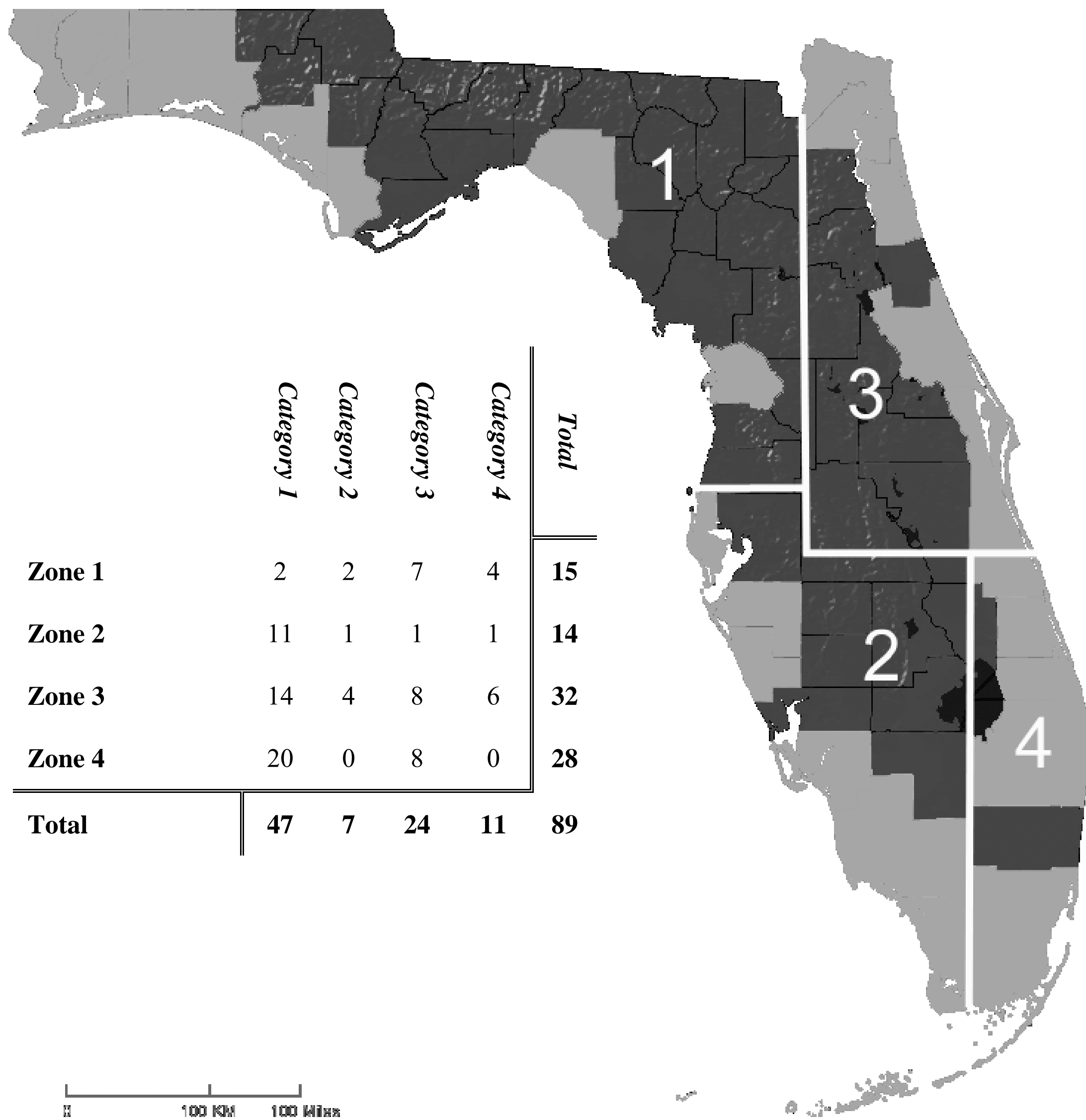
Spirorchiid trematodes and their associated cardiovascular lesions are among the oldest disease-related observations in the sea turtle literature (Looss, 1902). Over the last hundred years, significant progress has been made in the characterization of these parasites. There are, however, fundamental gaps in our understanding of their impact on sea turtle health, as our ability to study diseases is limited by difficultly in obtaining fresh carcasses, the investment required for thorough postmortem examination, and the advanced chronic state of disease that typifies many stranded turtles. This study is the product of a collaborative effort to investigate the basic aspects of the host-parasite relationship and the health implications of spirorchiidiasis in Florida turtles.

Prevalence of spirorchiid infection was high in examined *C. caretta* and *C. mydas* and was comparable to that observed by other investigators using similar methodology. The number of turtles of both species, especially adults, with spirorchiid-associated cardiovascular lesions of concern was notable. Several examples were observed in robust turtles with acute causes of death, which support that these parasites have the ability to infect and produce severe lesions in seemingly healthy turtles. It was surprising that more examples of fatal sequelae were not observed, as described in previous studies, given the extent of some lesions and apparent diminished integrity of affected arteries. Many turtles that develop fatal complications may die at sea and may not be well-represented in the stranding data.

The findings of this study contribute to both the diversity of spirorchiids known to parasitize Florida sea turtles and the spectrum of organs infected and associated pathological lesions. Many questions regarding the health implications of spirorchiidiasis remain. The effects of neurological infections and parasitism of endocrine organs, especially the thyroid

gland, require further investigation. The prevalence data and size class relationships identified herein hopefully will be used to guide and facilitate such studies.

Any comparisons between the findings of this study and previous reports require caution, as does extrapolation of these data to different sea turtles species, turtles of different size classes, or populations inhabiting different regions. The differences observed between host turtle species and among different size classes expand upon a very small number of studies that have considered such variables. The effort to understand spirorchiids and their implications on sea turtle health would greatly benefit from better definition of methodology, inclusion of biological host data (such as size class), and specific identification of parasites whenever possible to promote comparability among studies. At present, many generalizations are premature and much of the complexity of the host-parasite relationship remains to be revealed.



	Category 1	*Category 2*	*Category 3*	*Category 4*	***Total***
Zone 1	2	2	7	4	**15**
Zone 2	11	1	1	1	**14**
Zone 3	14	4	8	6	**32**
Zone 4	20	0	8	0	**28**
Total	**47**	**7**	**24**	**11**	**89**

Figure 2-1. Examined *C. caretta* by geographical region and category of necropsy data. Light gray areas are counties in which necropsied turtles were found stranded.

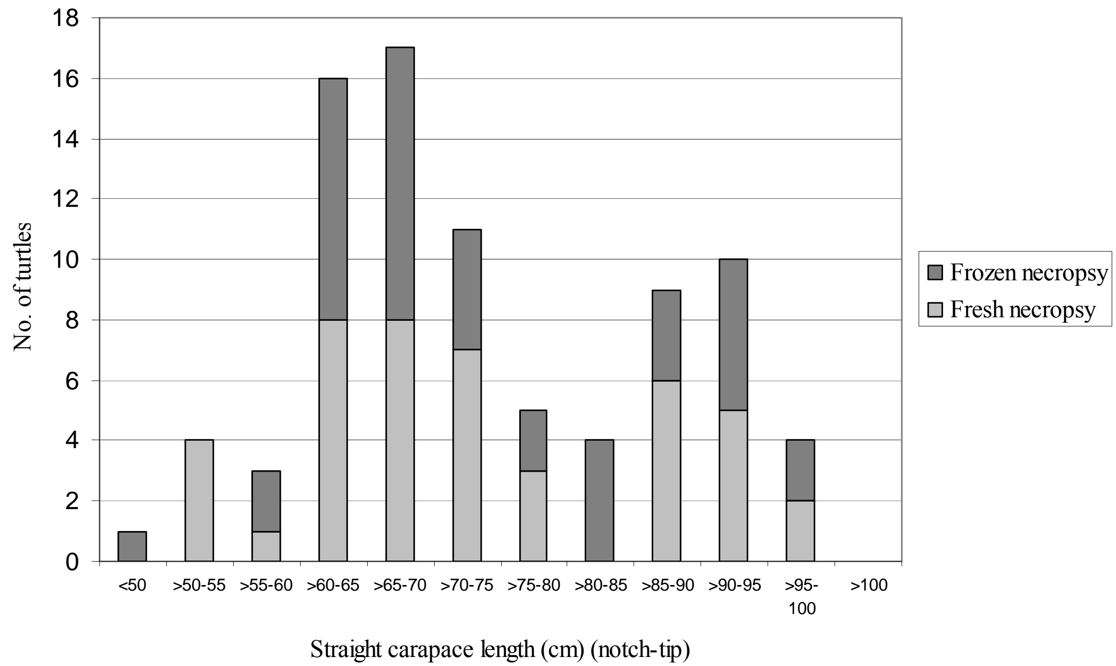
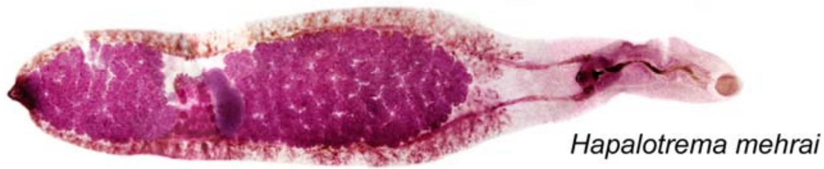


Figure 2-2. Histogram of size class frequencies of necropsied *Caretta caretta*. Fresh necropsies are category 1 turtles and frozen necropsies include all other categories.



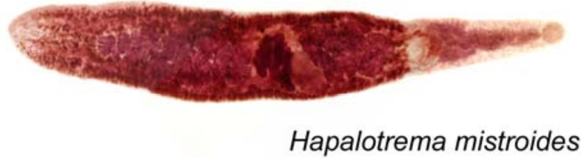


Figure 2-3. Photomicrographs of three *Hapalotrema* species collected from *C. caretta*.

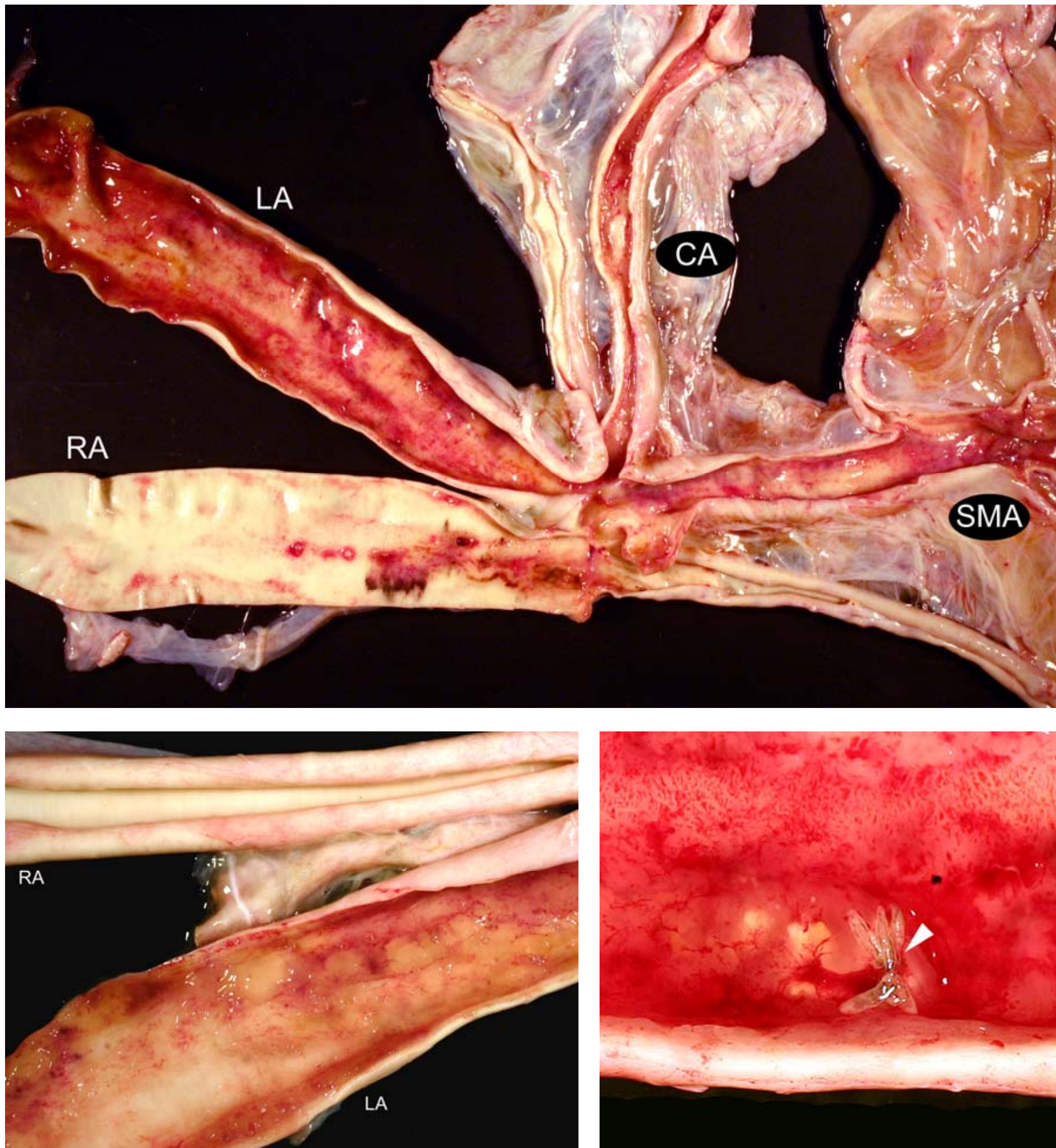


Figure 2-4. Arteritis associated with *Hapalotrema* infection in *C. caretta*. The top image depicts extensive thickening of the left aorta (LA), celiac artery (CA), superior mesenteric artery (SMA) and a congested, irregular endothelial surface (chronic arteritis). Multifocal lesions also are apparent within the right aorta (RA). In the lower left image, chronic arteritis of the left aorta (LA) is contrasted with a normal right aorta (RA). A cluster of *Hapalotrema mistroides* (white arrowhead) are attached within an area of endarteritis in the lower right image.

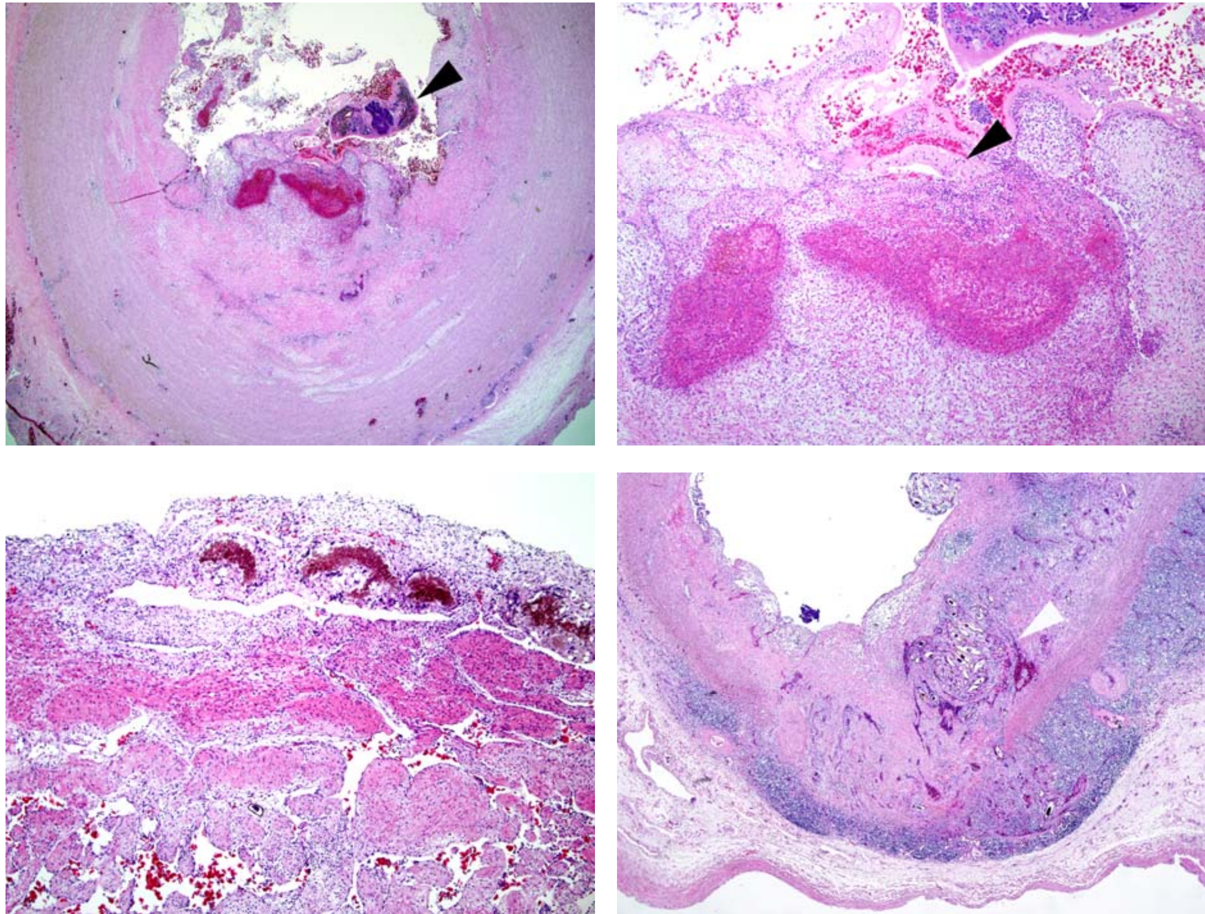


Figure 2-5. Photomicrographs of arteritis associated with *Hapalotrema* infection in *C. caretta*. The upper left image shows marked, diffuse thickening of the artery wall due to proliferation of the intima and a severe inflammatory infiltrate. A spirorchiid (*Hapalotrema mistroides*) is visible within the lumen (black arrowhead). At higher magnification (upper right image), an intense heterophilic infiltrate surrounds the acetabulum (black arrowhead) of a *H. mistroides*. More chronic lesions are shown in the lower images, which illustrate fibromuscular intimal proliferation and chronic granuloma formation with pigmented debris (left lower image), and a transmural infiltrate with intralesional spirorchiid egg granulomas (white arrowhead) and a cuff of mononuclear cells within the adventitia (lower right image).

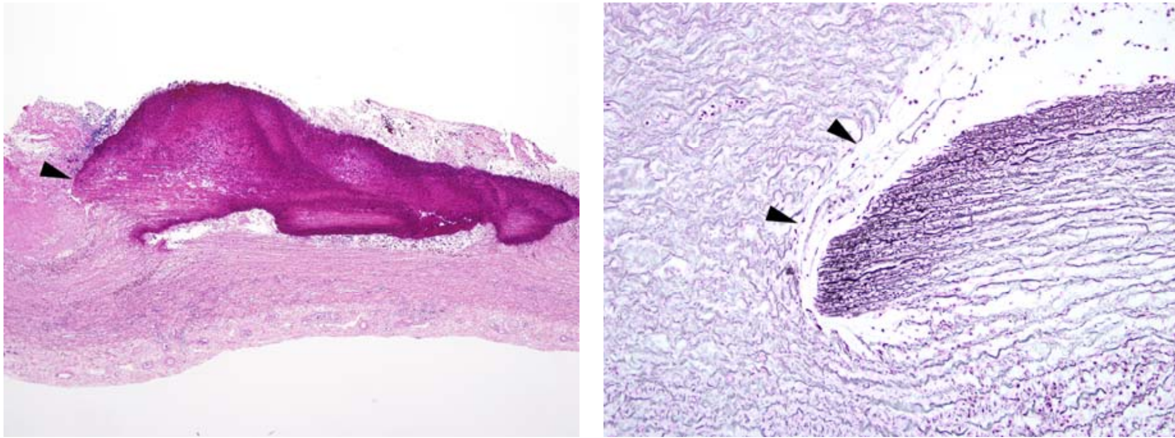


Figure 2-6. Necrosis of the tunica media in arteritis lesions associated with *Hapalotrema* infection in *C. caretta*. In the left image, there is extensive, well-demarcated necrosis of the tunica media (black arrowhead) underlying an area of endarteritis (H&E). In the right image, disruption of the reticulin fibers is visible at the margin of the necrotic segment (black arrowheads) (reticulin stain).

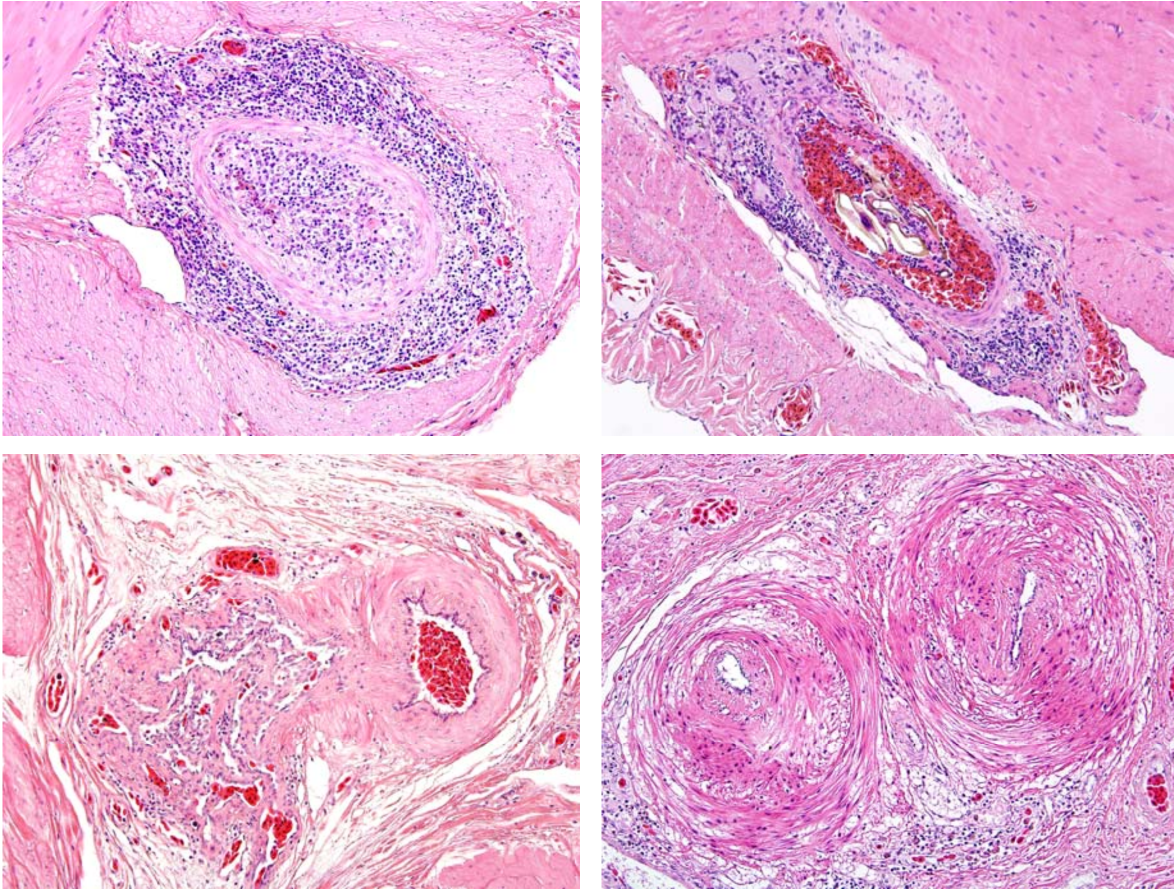


Figure 2-7. Histopathological lesions of small arteries (gastrointestinal tract) in *C. caretta* with spirorchiidiasis. The upper left image shows a small thrombosed artery with a mononuclear and granulocytic inflammatory infiltrate. The artery in the upper right image is similarly inflamed and has a small cluster of spirorchiid eggs (*Hapalotrema* sp.) within the lumen, as well as perivascular multinucleated giant cell formation. The lower left image demonstrates an area of microvascular proliferation in an animal that had other lesions, including arteritis and medial hypertrophy. The lower right image shows severe medial hypertophy of two small arteries.

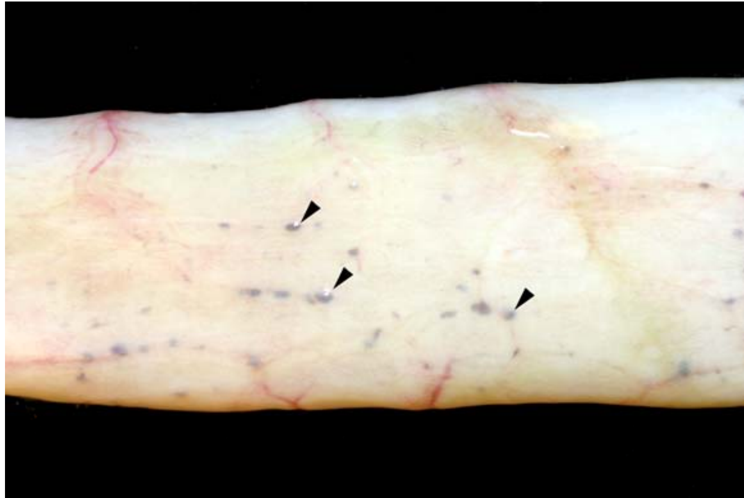


Figure 2-8. Masses of spirorchiid eggs (*Hapalotrema* sp.) within subserosal vessels of a *C. caretta*. This lesion is the typical appearance of subserosal egg masses observed in turtles infected with *Hapalotrema mistroides* or *H. pambanensis*.

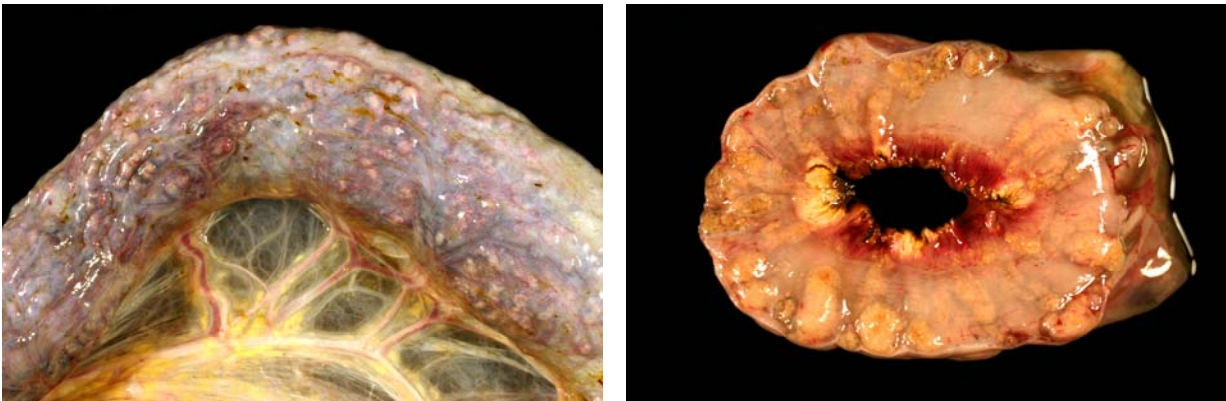


Figure 2-9. Extensive enteric lesions associated with *Hapalotrema* infection in a *C. caretta*. There are large transmural egg masses and thrombosis with ischemic necrosis of the enteric mucosa (right image) in this *C. caretta* infected with a novel species of *Hapalotrema*. These lesions are more extensive that those shown in Figure 2-8.

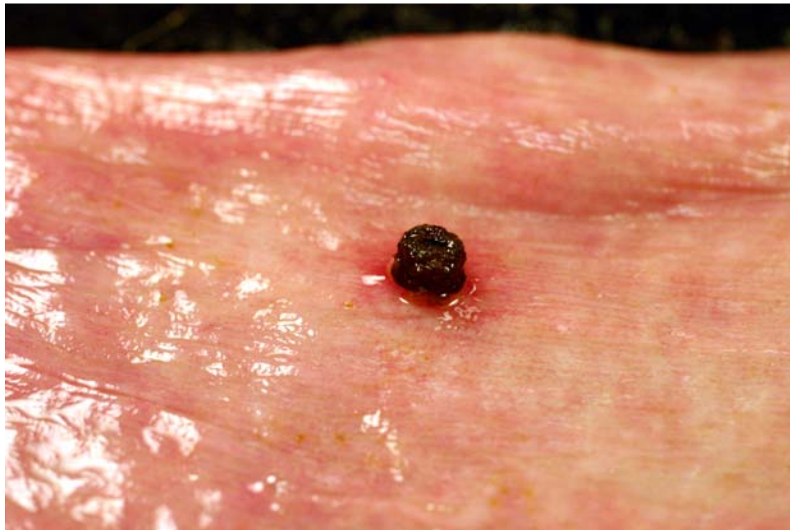


Figure 2-10. Colonic polyp in a *C. caretta* associated with granulomatous inflammation and intralesional *Hapalotrema* eggs.

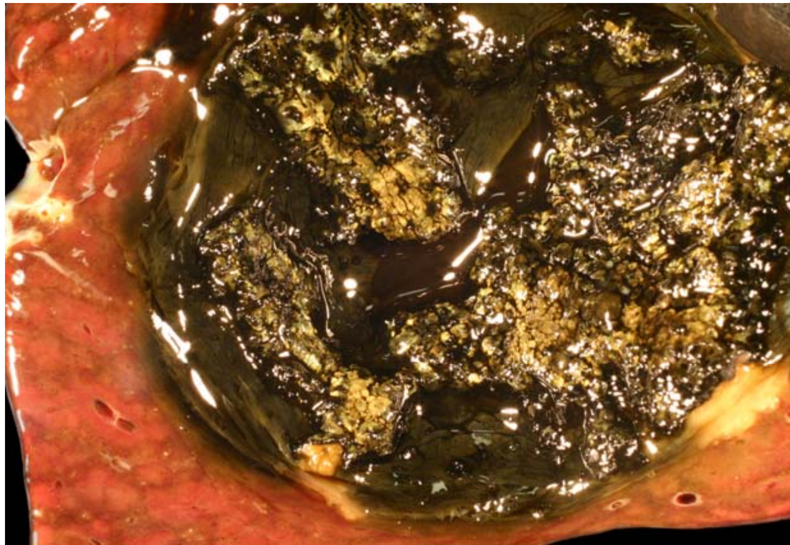


Figure 2-11. Granulomatous cholecystitis with papillary mucosal proliferation in a *C. caretta* associated with *Hapalotrema* eggs (*Hapalotrema* nov. sp.)

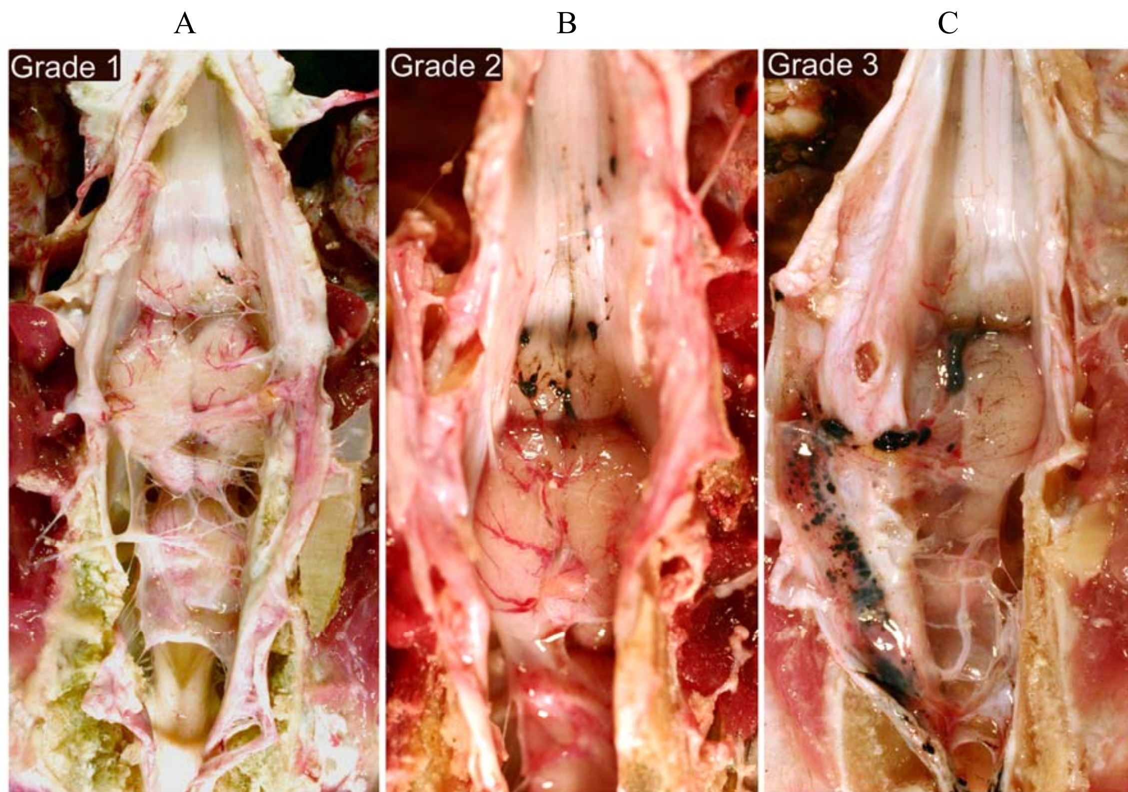


Figure 2-12. Comparison of grades of neurospirorchiidiasis (*Neospirorchis* sp.) in *C. caretta*. A) Only two small egg masses, both less than 3 mm in diameter. B) Approximately eight to ten distinct egg masses, all of which are less than 3 mm in diameter. C) Greater than 10 egg masses, including several large, coalescing masses that distend meningeal vessels.

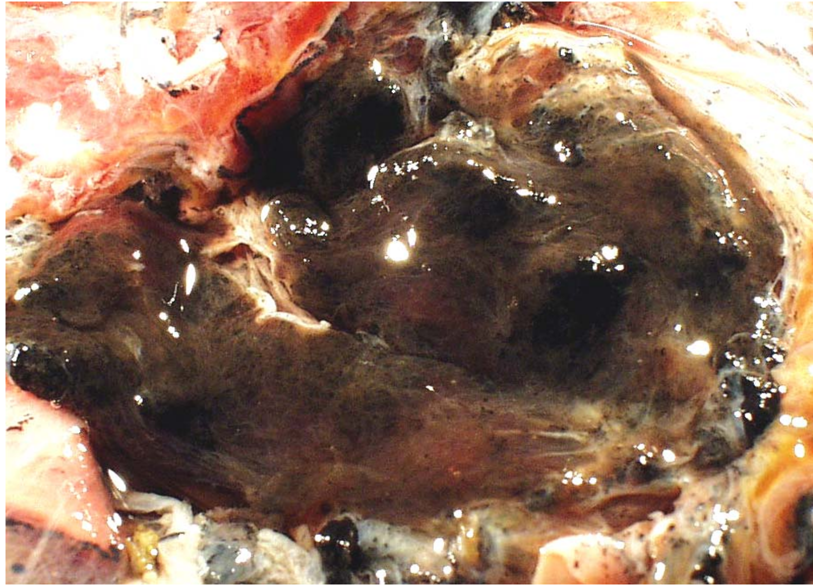


Figure 2-13. Severe infection of the thyroid gland by *Neospirorchis* sp. in *C. caretta*. Large black masses of spirorchiid egges efface the gland and distend blood vessels within the surrounding cervical tissues.

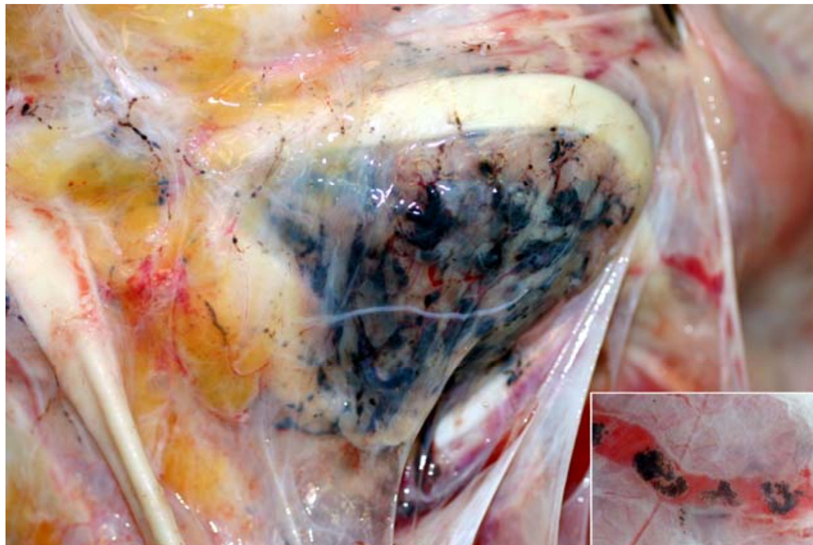


Figure 2-14. Occlusion of thymic blood vessels by eggs of *Neospirorchis* sp. in *C. caretta*. Large black spirorchiid egg masses are seen within thymic vessels and egg emboli are demonstrated within the higher magnification inset.

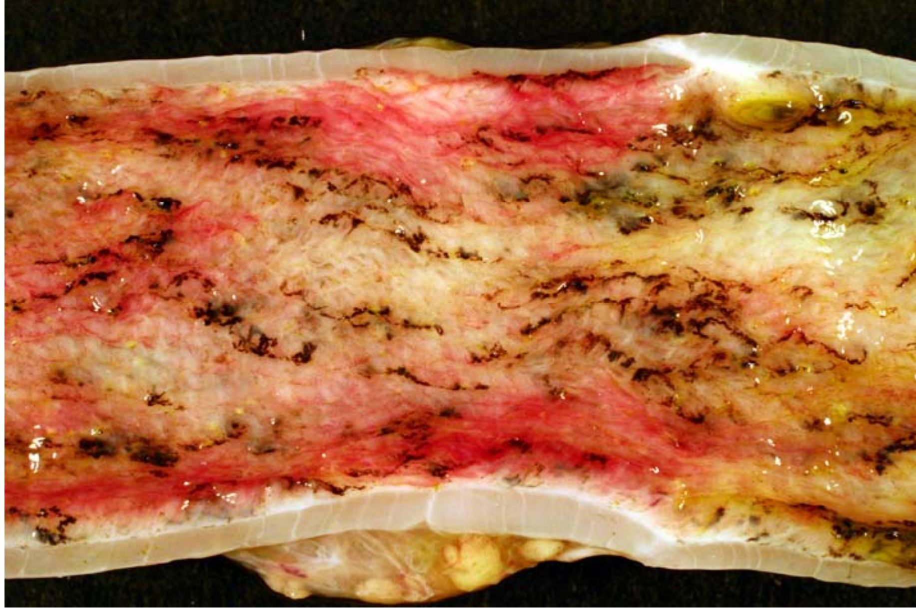


Figure 2-15. Abundant submucosal egg masses (*Neospirorchis* sp) in the small intestine of a *C. caretta*. Numerous black, serpiginous egg masses are visible within the submucosa.

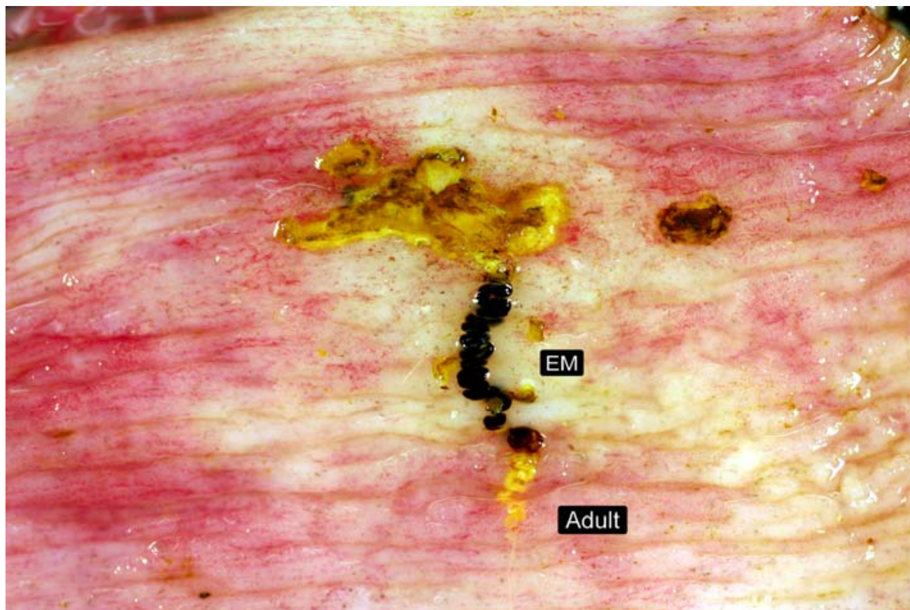


Figure 2-16. Enteric ulceration secondary to spirorchiidiasis (*Neospirorchis* sp.) in *C. caretta*. In this image, an ulcer is forming along the path of a large egg mass (EM). The filiform adult spirorchiid can be seen migrating through a submucosal vessel and the anterior portion has been freed from the tissue and is laying on the mucosal surface.

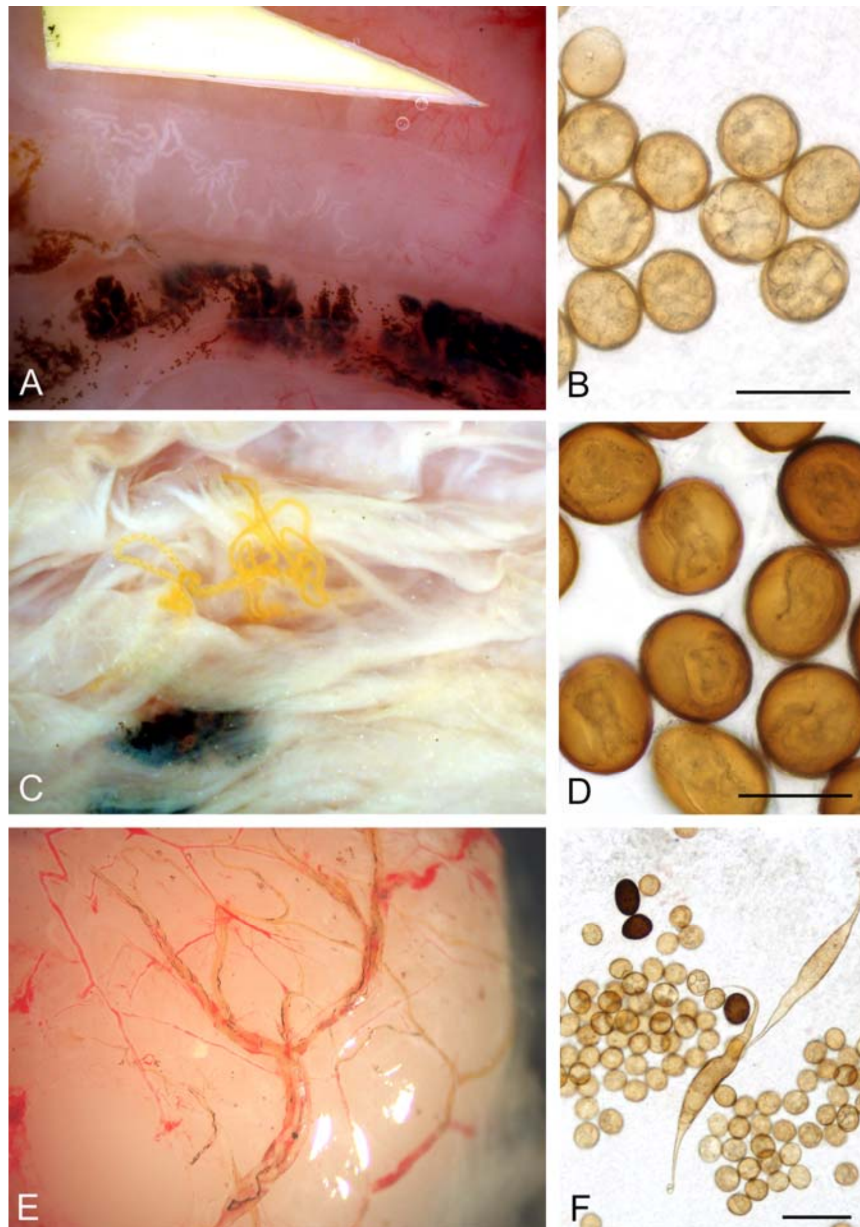


Figure 2-17. Images illustrating variation in adult and egg morphology among observed specimens of *Neospirorchis* from *C. caretta*. The upper (A) and middle (C) images show *Neospirorchis* specimens adjacent to egg masses within the enteric submucosa. Example A is more petite and white as compared to the larger and yellow example C. The eggs in the adjacent images (B and F) correspond to the adult morphological types and demonstrate the smaller egg size for the adult in A (both bars = 50 µm). In addition, the *Neospirorchis* in the leptomeninges (E) have prominent pigmented cecae, which are not observed in the two gastrointestinal examples. Figure F demonstrates variation in *Neospirorchis* egg morphology in a squash preparation from the lung of an infected *C. caretta* (bar = 100 µm). Two *Hapalotrema* eggs with bipolar processes also are present.

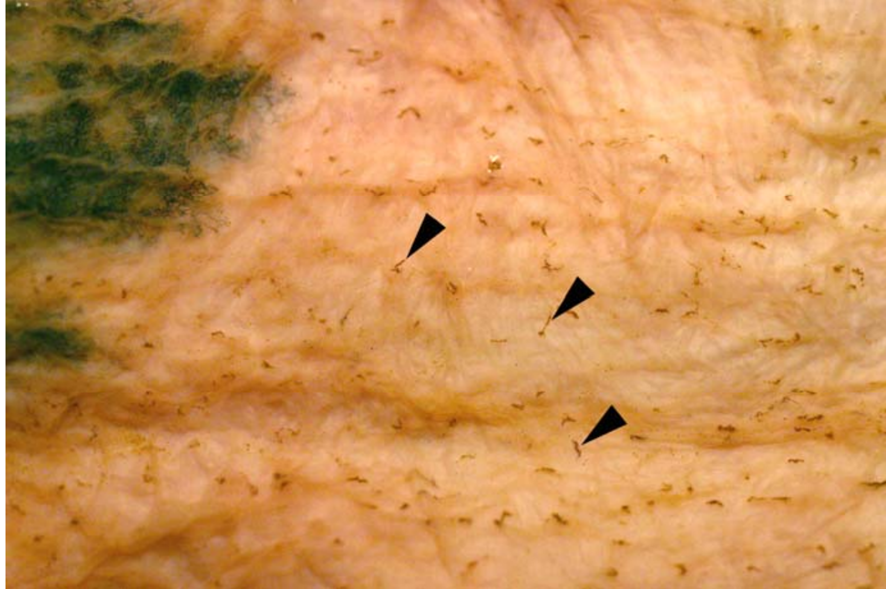


Figure 2-18. Spirorchiid egg masses (*Neospirorchis* sp.) within cloacal submucosal vessels of a *C. caretta*. Each cluster (black arrowheads) measures approximately 1.0 mm in diameter.

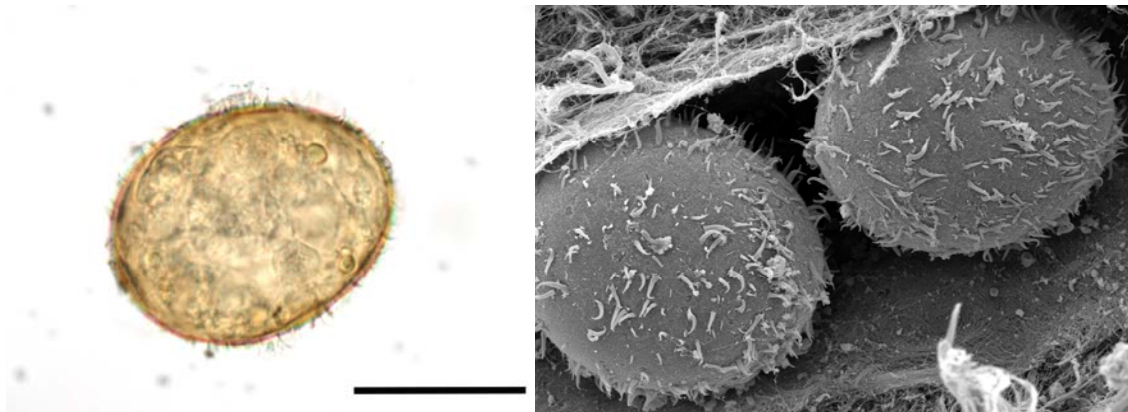


Figure 2-19. Cytology and scanning electron microscopy (SEM) of cloacal *Neospirorchis* egg masses from a *C. caretta*. Cytological impressions (left image) of the egg masses from Figure 2-18 reveal fine surface projections on the shell surface (bar = 25 µm). These projections are readily observed by scanning electron microscopy (lower right)

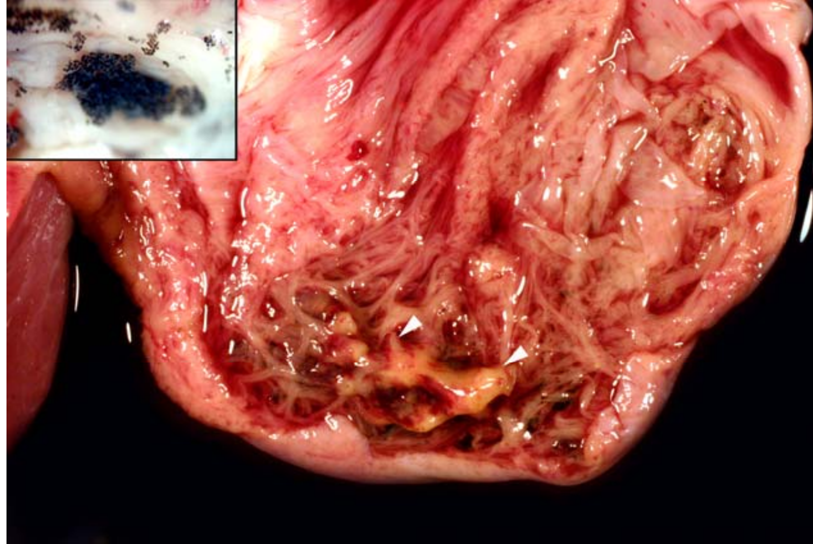


Figure 2-20. Atrial thrombosis associated with *Neospirorchis pricei* infection in a *C. caretta*. The left atrium has been opened and a thrombus (white arrowheads) is adhered to the endocardium. The higher magnificantion inset shows the dense aggregates of *Neospirorchis* eggs underlying the thrombus.

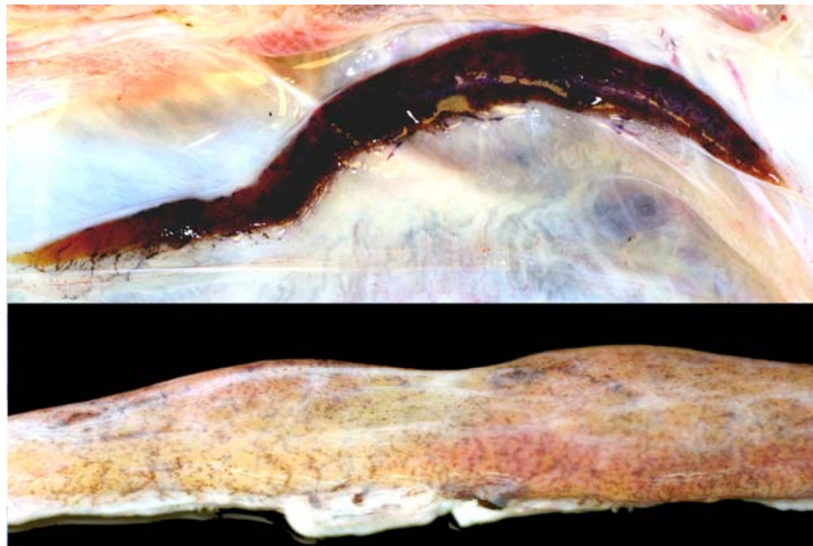
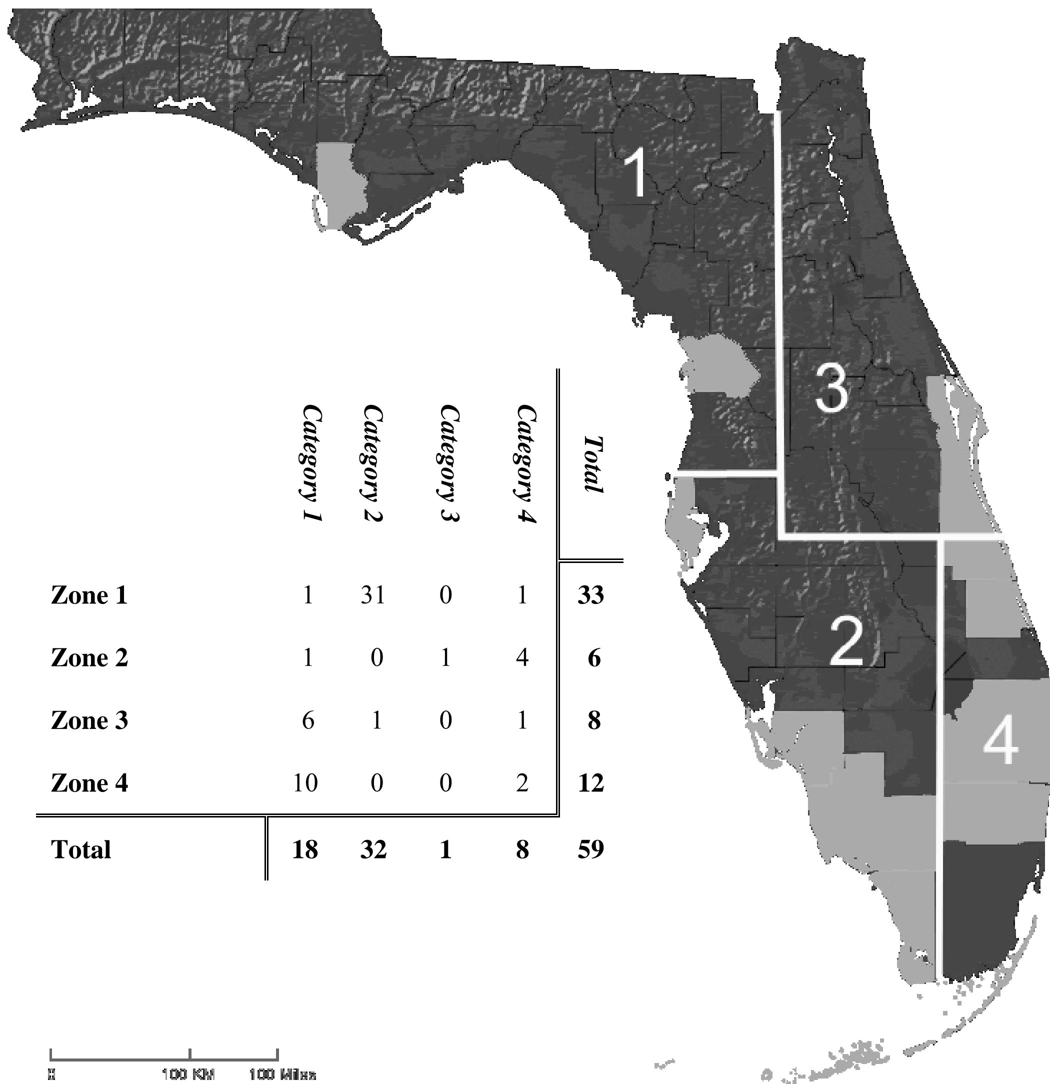


Figure 2-21. Severe verminous orchitis in a *C. caretta* infected with *Neospirorchis* sp.. Numerous *Neospirorchis* eggs and adults (not visible) are distributed throughout the testis. Shown are the testis in situ (top) in which eggs are observed within adjacent vessels. Individual eggs are more easily observed in the closer image (bottom).



	Category 1	*Category 2*	*Category 3*	*Category 4*	***Total***
Zone 1	1	31	0	1	**33**
Zone 2	1	0	1	4	**6**
Zone 3	6	1	0	1	**8**
Zone 4	10	0	0	2	**12**
Total	**18**	**32**	**1**	**8**	**59**

Figure 2-22. Examined *C. mydas* by geographical region and category of necropsy data. Light gray areas are counties in which necropsied turtles were found stranded.

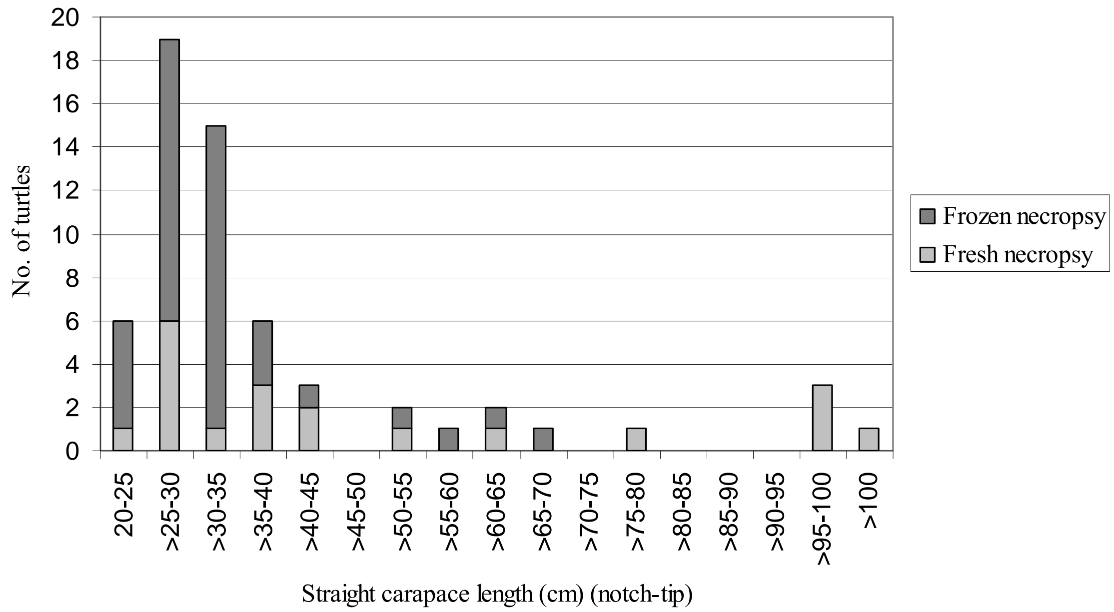


Figure 2-23. Histogram of size class frequencies of necropsied *C. mydas*. Fresh necropsies are category 1 turtles and frozen necropsies include all other categories.

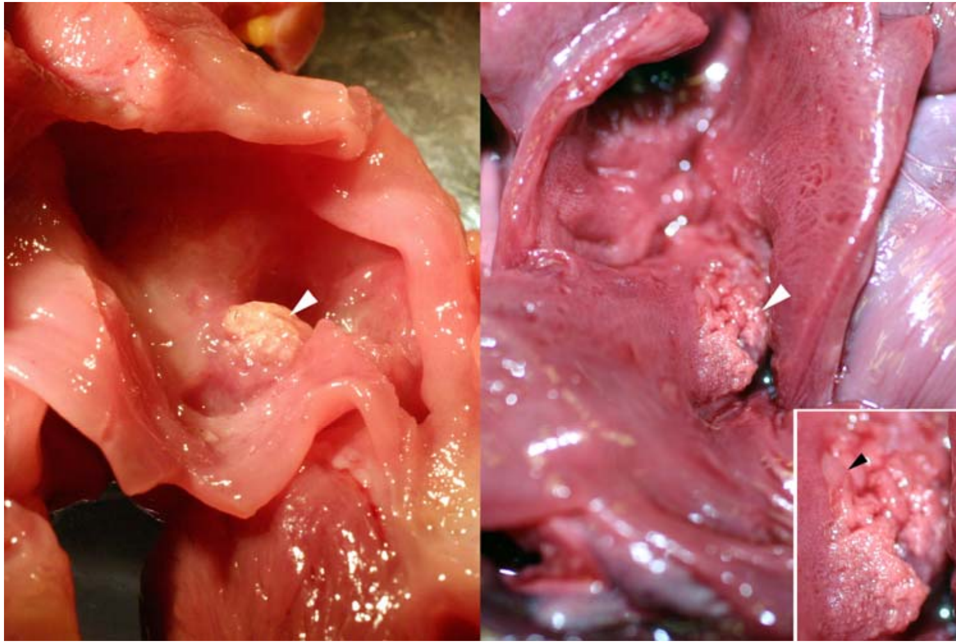


Figure 2-24. Endocarditis in *C. mydas* associated with *L. learedi* infection. The atria have been opened in both photographs exposing the supravalvular endocardium. Both cases have well-demarcated, raised areas of endocarditis (white arrows). An adult spirorchiid (*Learedius learedi*) (black arrowhead) can be seen attached to the lesion in closer image (inset).

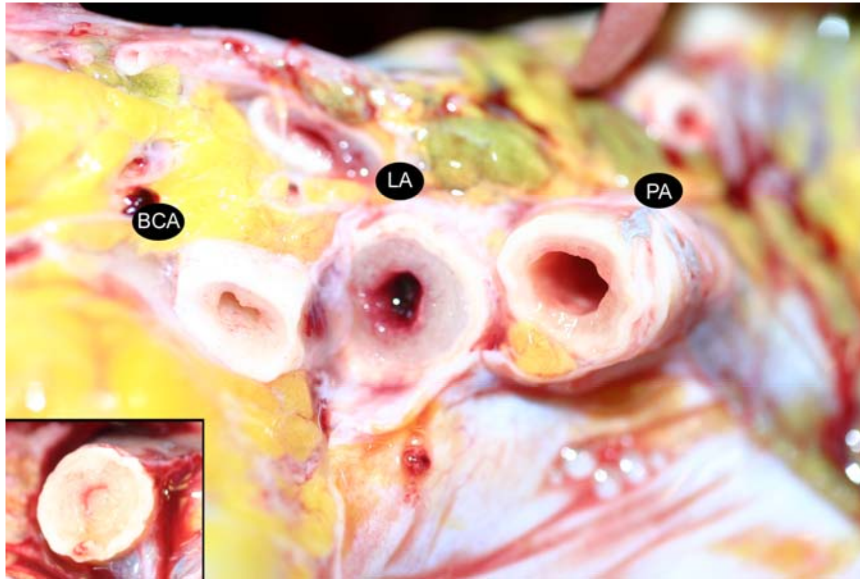


Figure 2-25. Severe arteritis in a *C. mydas* associated with *L. learedi* infection. There is severe thickening of the left brachiocephalic artery (BCA), left aorta (LA), and left pulmonary artery (PA). The lumina are severely reduced, especially in the left aorta and brachiocephalic artery (inset), due to intimal proliferation and chronic arteritis.

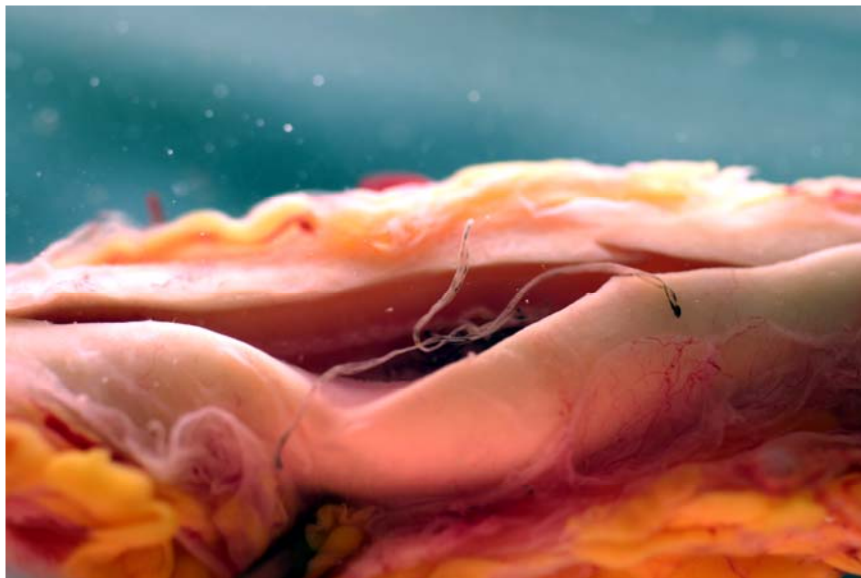


Figure 2-26. Arteritis in a *C. mydas* associated with *H. postorchis* infection. The artery wall is severely thickened and three adult spirorchiids (*Hapalotrema postorchis*) are seen protruding from the vessel lumen.

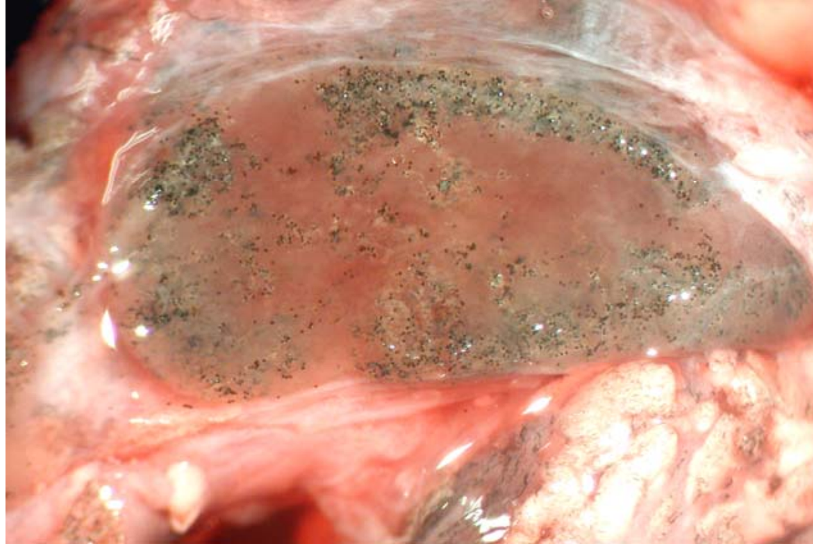


Figure 2-27. Thyroiditis in *C. mydas* associated with infection by *Neospirorchis* sp: Numerous spirorchiid eggs (*Neospirorchis* sp.) and associated granulomatous inflammation are distributed throughout the thyroid gland.

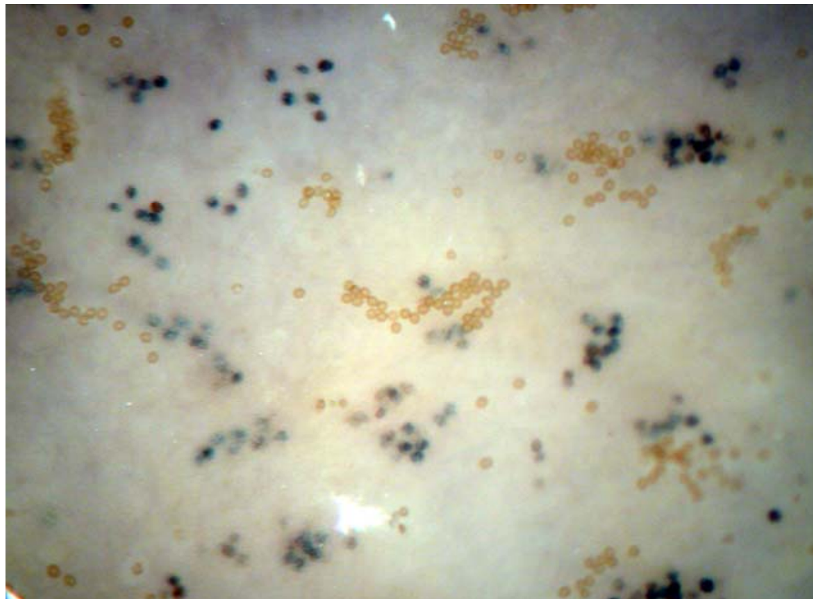


Figure 2-28. Eggs of *Neospirorchis* species within the gastric submucosa of a *C. mydas*. Two morphologies of *Neospirorchis* eggs are observed. The larger, dark brown eggs are embolized deep within the submucosa. The smaller, golden eggs are dispersed into loosely organized clusters within the superficial submucosa.

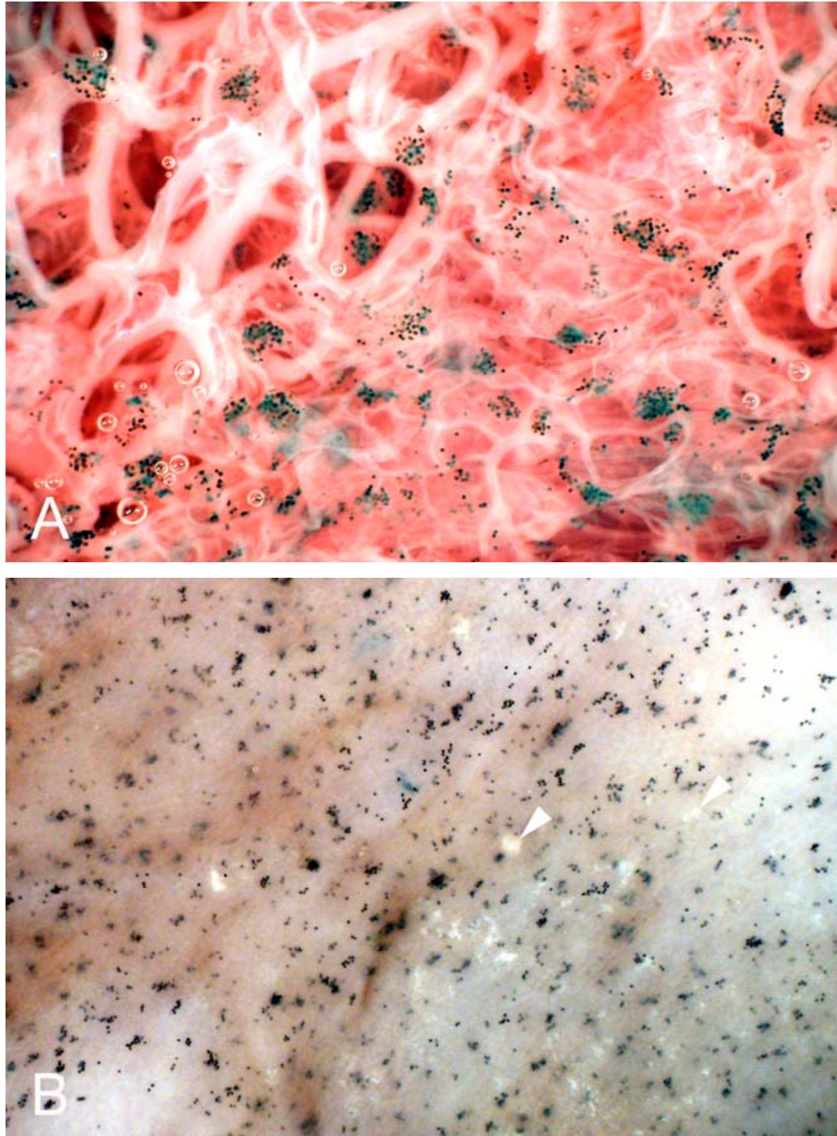


Figure 2-29. Numerous embolized *Neospirorchis* eggs in the lungs and gastric mucosa of a *C. mydas*. Numerous *Neospirorchis* eggs are embolized throughout the lungs (A), stomach (B), and other organs in these *C. mydas* that died during a hypothermic stunning event. Note the multifocal mucosal erosion and inflammation (white arrowheads) in the lower image.

Table 2-1. Criteria for categorization of the duration of illness.

Category	Criteria for classification
Acute insult	1. Carcass in good nutritional condition based on musculature (no atrophy) and adipose stores (minimal or no atrophy present). 2. Any identifiable fatal pathological lesion is characterized by an acute inflammatory response or other cellular injury.
Intermediate insult	1. Atrophy of skeletal muscle is present or absent, adipose tissue is atrophied, bone density is within normal limits, no catabolism of connective tissues. 2. Any identifiable fatal pathological lesion is characterized by a chronic inflammatory response or other cellular injury and fibrosis.
Chronic insult	1. Carcass exhibits advanced atrophy of skeletal muscle and adipose tissue (emaciation). Osteopenia and catabolism of connective tissues present. 2. Any identifiable fatal pathological lesion is characterized by a chronic inflammatory response or other cellular injury and fibrosis.

Table 2-2. Criteria for determining spirorchiid trematode impact score.

Score	Definition and criteria
1	None detected.
2	Incidental infection. Numbers of adult spirorchiids, if detected, were low (4 or less) and egg deposition in tissues consisted either of rare individual embolized eggs and/or isolated egg masses. Associated tissue/organ injury or inflammation is mild and focal in distribution.
3	Contributory. Large numbers of adult spirorchiids and/or eggs are associated with significant organ injury. Included in this category are turtles in which severe, multisystemic spirorchiid egg embolization is one of multiple significant pathological findings. Also included are turtles in which injury/inflammation associated with spirorchiids has resulted in or exacerbated other conditions, including thrombosis and secondary bacteria infection.
4	Fatal. The severity and extent of organ/tissue injury or inflammation associated with spirorchiid adults or eggs supports spirorchiidiasis is the cause of death.
5	Unknown. Large numbers of adult spirorchiids and/or eggs are present and/or there is significant associated organ injury, but significance cannot be confidently determined from necropsy data. Included in this group are turtles with a known cause of death in which association, if any, with spirorchiidiasis is unknown.

Table 2-3. Criteria for grading spirorchiid infection and associated pathological lesions.

I. Arteritis of large arteries associated with *Hapalotrema* infection

Grade	Criteria for grading
0	No gross lesions Histological correlate: Within normal limits
1	Focal gross lesion (≤1.5 cm diameter) Histological correlate: inimal or mild subintimal inflammatory infiltrate and/or fibrosis
2	Focally extensive (1.5 cm to ≤4.0 cm in greatest dimension) OR multifocal: maximum of 3 lesions (any single lesion ≤2.0 cm diameter or ≤4.0 cm in greatest dimension) Histological correlate: Moderate changes including inflammation and intimal proliferation
3	Regionally extensive (single or multiple) (>4.0 cm in any dimension) OR Diffuse lesions involving one or more vessels Histological correlate: Changes as under 2 but severe and/or any one of the following: thrombus formation, aneurysm formation, necrosis of tunica media

II. Neurospirorchiidiasis (*Neospirorchis* species)

Grade	Criteria for grading
0	No egg masses or adults / individual embolized eggs only
1	Small numbers: typically 1-5 egg masses measuring less than 3 mm in greatest dimension and/or small numbers (1-3) adults
2	Moderate numbers: typically 5 -10 egg masses with most measuring less than 3 mm in greatest dimension and/or 1-2 larger coalescing egg masses distending meningeal vessels
3	Large numbers: >10 egg masses with regionally intense or diffuse distribution and/or >2 larger coalescing egg masses distending meningeal vessels

III. Parasitism of the thyroid gland and thymus (*Neospirorchis* species)

Grade	Criteria for grading
0	No eggs OR rare to small numbers of embolized eggs only
1	Small numbers of adults and/or egg masses
2	Moderate numbers (includes focally extensive aggregates and diffuse lesions) and/or adults
3	Large numbers (large areas of gland/thymus effaced by eggs and associated inflammation)

IV. Enteric spirorchiidiasis (*Neospirorchis* species)

Grade	Criteria for grading
0	No egg masses
1	1 – 10 distinct egg mass
2	>10 – 50 distinct egg masses
3	>50 distinct egg masses

Table 2-4. Causes of death in *C. caretta* categorized by duration of illness (category 1 & 2 data).

Primary Diagnosis/ Cause of Death	Acute	Intermediate	Chronic	Total
Traumatic injury	6	2	-	8
Brevetoxicosis	7	-	-	7
Unusual mortality event of unknown etiology	4	6	-	10
Drowning / aspiration	2	-	-	2
Infectious disease	-	5	2	7
Enteric impaction	-	1	2	3
Emaciation	-	-	3	3
Multi-factorial	-	-	13	13
Undetermined	1	-	-	1
Total	20	14	20	54

Tabe 2-5. Prevalence of *Hapaltrema*-associated large vessel arteritis in *C. caretta* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category				
	Category 1	Category 2	Category 3	Total
Grade 0	51.1% (24/47)	85.7% (6/7)	79.2% (19/24)	62.8% (49/78)
Grade 1	8.5% (4/47)	0% (0/7)	4.2% (1/24)	6.4% (5/78)
Grade 2	12.8% (6/47)	14.3% (1/7)	12.5% (3/24)	12.8% (10/78)
Grade 3	27.7% (13/47)	0% (0/7)	4.2% (1/24)	17.9% (14/78)

Duration of illness			
	Acute	Intermediate	Chronic
Grade 0	64.0% (16/25)	66.7% (12/18)	60.0% (21/35)
Grade 1	12.0% (3/25)	5.6% (1/18)	2.9% (1/35)
Grade 2	16.0% (4/25)	5.6% (1/18)	14.3% (5/35)
Grade 3	8.0% (2/25)	22.2% (4/18)	22.9% (8/35)

Size class (Straight carapace length)			
	≤65 cm	>65-85 cm	>85 cm
Grade 0	66.7% (14/21)	77.1% (27/35)	14.3% (2/14)
Grade 1	9.5% (2/21)	0% (0/35)	14.3% (2/14)
Grade 2	9.5% (2/21)	11.4% (4/35)	21.4% (3/14)
Grade 3	14.3% (3/21)	11.4% (4/35)	50.0% (7/14)

Table 2-6. Anatomic locations from which adult specimens of *Hapalotrema* species were collected from *C. caretta*.

Species	Heart	Aortas	Mesenteric a.	Hepatic vessels
H. mistroides	4	11	8	1
H. pambanensis	3	2	0	1
Hapalotrema. sp. (novel)	0	0	2	1

Each cell indicates the number of individual turtles from which a given species was recovered from a specific site, not the number of parasites.

Table 2-7. Prevalence of *Neospirorchis* sp. by anatomic location (adults and/or localized egg deposition) and necropsy data category.

Location	Category 1	Category 2	Category 3	Category 4	Total
Meninges	51.1% (24/47)	42.9% (3/7)	54.2% (13/24)	36.4% (4/11)	49.4% (44/89)
Thyroid gland	57.4% (27/47)	0% (0/7)	85.7% (18/21)	-	60.0% (45/75)
Thymus	46.8% (22/47)	28.6% (2/7)	59.1% (13/22)	-	48.7% (37/76)
Gastrointestinal	66.0% (31/47)	71.4% (5/7)	63.6% (14/22)	-	65.8% (50/76)
Heart/major arteries	12.8% (6/47)	0% (0/7)	-	-	11.1% (6/54)
Adrenal glands	31.9% (15/47)	33.3% (1/3)	-	-	32.0% (16/50)
Testes	25.0% (3/12)[a]	-	-	-	25.0% (3/12)

[a]Only males included in prevalence.

Table 2-8. Prevalence of neurospirorchiidiasis (*Neospirorchis* sp.) in *C. caretta* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category					
	Category 1	Category 2	Category 3	Category 4	Total
Grade 0	45.7% (21/46)	57.1% (4/7)	45.8% (11/24)	63.6% (7/11)	48.9% (43/88)
Grade 1	34.8% (16/46)	42.9% (3/7)	33.3% (8/24)	18.2% (2/11)	32.9% (29/88)
Grade 2	13.0% (6/46)	0% (0/7)	20.8% (5/24)	0% (0)	12.5% (11/88)
Grade 3	6.5% (3/46)	0% (0/0)	0% (0)	18.2% (2/11)	5.7% (5/88)

Duration of illness			
	Acute	Intermediate	Chronic
Grade 0	50.9% 16/32)	55.6% (10/18)	44.7% (17/38)
Grade 1	40.6% (13/32)	22.2% (4/18)	31.6% (12/38)
Grade 2	3.1% (1/32)	22.2% (4/18)	15.8% (6/38)
Grade 3	6.3% (2/32)	0% (0/18)	7.9% (3/38)

Size class (Straight carapace length)			
	≤65 cm	>65-85 cm	>85 cm
Grade 0	80.0% (20/25)	41.0% (16/39)	11.8% (2/17)
Grade 1	16.0% (4/25)	33.3% (13/39)	52.9% (9/17)
Grade 2	4.0% (1/25)	17.9% (7/39)	23.5% (4/17)
Grade 3	0% (0/25)	7.7% (3/39)	11.8% (2/17)

Table 2-9. Prevalence of thyroid gland parasitism (*Neospirorchis* sp.) in *C. caretta* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category				
	Category 1	Category 2	Category 3	Total
Grade 0	42.6% (20/47)	100% (7/7)	33.3% (7/21)	45.3% (34/75)
Grade 1	23.4% (11/47)	0% (0/7)	19.0% (4/21)	20.0% (15/75)
Grade 2	23.4% (11/47)	0% (0/7)	28.6% (6/21)	22.7% (17/75)
Grade 3	10.6% (5/47)	0% (0/7)	19.0% (4/21)	12.0% (9/75)
Duration of illness				
	Acute	Intermediate	Chronic	
Grade 0	68.0% (17/25)	50.0% (9/16)	26.5% (9/34)	
Grade 1	16.0% (4/25)	31.3% (5/16)	17.6% (6/34)	
Grade 2	16.0% (4/25)	12.5% (2/16)	32.4% (11/34)	
Grade 3	0% (0/25)	6.2% (1/16)	23.5% (8/34)	
Size class (Straight carapace length)				
	≤65 cm	>65-85 cm	>85 cm	
Grade 0	45.0% (9/20)	30.3% (10/33)	57.1% (8/14)	
Grade 1	15.0% (3/20)	24.2% (8/33)	21.4% (3/14)	
Grade 2	25% (5/20)	27.3% (9/33)	21.4% (3/14)	
Grade 3	15.0% (3/20)	18.2% (6/33)	0% (0/14)	

Table 2-10. Prevalence of parasitism (*Neospirorchis* sp.) of the thymus in *C. caretta* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category				
	Category 1	Category 2	Category 3	Total
Grade 0	53.2% (25/47)	71.4% (5/7)	40.1% (9/22)	51.3% (39/76)
Grade 1	23.4% (11/47)	28.6% (2/7)	36.4% (8/22)	27.6% (21/76)
Grade 2	8.5% (4/47)	0% (0/7)	9.1% (2/22)	7.9% (6/76)
Grade 3	14.9% (7/47)	0% (0/7)	13.6% (3/22)	13.2% (10/76)
Duration of illness				
	Acute	Intermediate	Chronic	
Grade 0	84.0% (21/25)	56.3% (9/16)	25.7% (9/35)	
Grade 1	16.0% (4/25)	31.3% (5/16)	34.3% (12/35)	
Grade 2	0% (0/25)	6.3% (1/16)	14.3% (5/35)	
Grade 3	0% (0/25)	6.3% (1/16)	25.7% (9/35)	
Size class (Straight carapace length)				
	≤65 cm	>65-85 cm	>85 cm	
Grade 0	40.0% (8/20)	44.1% (15/34)	78.6% (11/14)	
Grade 1	25.0% (5/20)	26.5% (9/34)	21.4% (3/14)	
Grade 2	15.0% (3/20)	11.8% (4/34)	0% (0/14)	
Grade 3	20.0% (4/20)	17.6% (6/34)	0% (0/14)	

Table 2-11. Prevalence of enteric *Neospirorchis* sp. in *C. caretta* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category			
	Category 1	Category 2	Total
Grade 0	38.6% (17/44)	33.3% (2/6)	38.0% (19/50)
Grade 1	31.8% (14/44)	66.7% (4/6)	36.0% (18/50)
Grade 2	20.5% (9/44)	0% (0/6)	18.0% (9/50)
Grade 3	11.4% (5/44)	0% (0/6)	10.0% (5/50)
Duration of illness			
	Acute	Intermediate	Chronic
Grade 0	66.7% (12/18)	35.7% (5/14)	11.1% (2/18)
Grade 1	22.2% (4/18)	42.9% (6/14)	44.4% (8/18)
Grade 2	11.1% (2/18)	21.4% (3/14)	22.2% (4/18)
Grade 3	0% (0/18)	0% (0/14)	22.2% (4/18)
Size class (Straight carapace length)			
	≤65 cm	>65-85 cm	>85 cm
Grade 0	46.2% (6/13)	20.0% (3/15)	71.4% (10/14)
Grade 1	46.2% (6/13)	20.0% (3/15)	21.4% (3/14)
Grade 2	7.7% (1/13)	40.0% (6/15)	7.1% (1/14)
Grade 3	0% (0/13)	20.0% (3/15)	0% (0/14)

Table 2-12. Spirorchiid impact ratings (SIR) in *C. caretta* categorized by duration of illness.

	SIR 1	SIR 2	SIR 3	SIR 4	SIR 5
Acute	1	15	0	0	4
Intermediate	1	6	1	0	6
Chronic	0	6	5	1	8
Total	2	27	6	1	18

Table 2-13. Causes of death in necropsied *C. mydas* by duration of insult.

Primary Diagnosis/ Cause of Death	Acute	Intermediate	Chronic	Total
Hypothermic stunning	30	-	-	30
Traumatic injury	7	3	1	11
Fibropapillomatosis	-	2	1	3
Infectious disease	-	3	1	4
Emaciation	-	-	1	1
Multifactorial	-	-	1	1
Total	37	8	5	50

Table 2-14. Prevalence of *Neospirorchis* sp. in *C. mydas* by anatomic location (adults and/or localized egg deposition) and necropsy data category.

Location	Category 1	Category 2	Category 3	Category 4	Total
Meningeal	22.2% (4/18)	0% (0/32)	0% (0/1)	37.5% (3/8)	11.9% (7/59)
Thyroid gland	22.2% (4/18)	40.6% (13/32)	0% (0/1)		33.3% (17/51)
Gastric	22.2% (4/18)	3.3% (1/30)[a]	-		10.4% (5/48)

[a]Subgross examination not performed in omitted animals.

Table 2-15. Prevalence of thyroid gland parasitism (*Neospirorchis* sp.) in *C. mydas* by grade, necropsy data category, duration of illness, and size class.

Necropsy data category				
	Category 1	Category 2	Category 3	Total
Grade 0	77.8% (14/18)	59.4% (19/32)	100% (1/1)	66.7% (34/51)
Grade 1	11.1% (2/18)	18.8% (6/32)	0% (0/1)	15.7% (8/51)
Grade 2	5.6% (1/18)	15.6% (5/32)	0% (0/1)	11.8% (6/51)
Grade 3	5.6% (1/18)	6.3% (2/32)	0% (0/1)	5.9% (3/51)
Duration of illness				
	Acute	Intermediate	Chronic	
Grade 0	57.9% (22/38)	100% (8/8)	80.0% (4/5)	
Grade 1	21.0% (8/38)	0% (0/8)	0% (0/5)	
Grade 2	15.8% (6/38)	0% (0/8)	0% 0/5)	
Grade 3	5.3% (2/38)	0% (0/8)	20.0% (1/5)	
Size class (Straight carapace length)				
	≤65	>65-85	>85	
Grade 0	63.8% (30/47)	100% (1/1)	75% (3/4)	
Grade 1	17.0% (8/47)	48.5% (0/1)	0% (0/0)	
Grade 2	10.6% (5/47)	27.3% (0/1)	25.0% (1/4)	
Grade 3	8.5% (4/47)	18.2% (0/1)	0% (0/0)	

Table 2-16. Spirorchiid impact ratings (SIR) in *C. mydas* categorized by duration of illness.

	SIR 1	SIR 2	SIR 3	SIR 4	SIR 5
Acute	4	24	0	0	9
Intermediate	0	7	1	0	0
Chronic	0	4	0	0	1
Total	4	35	1	0	10

Table 2-17. Body condition indices (BCI's) and *Neospirorchis*-related findings in hypothermic-stunned immature *C. mydas* – St. Joseph Bay, Florida.

Necropsy No.	BCI (g/cm^3)	BCI (g/cm)	Spirorchiid-associated findings
GST0801	1.33	0.116	None present
GST0802	1.16	0.112	Moderate numbers embolized *Neospirorchis* eggs in lungs
GST0803	1.27	0.145	Adult *Neospirorchis* in thyroid gland
GST0804	1.25	0.106	None present
GST0805	1.17	0.104	Moderate numbers *Neospirorchis* eggs in thyroid; moderate embolization to lungs and stomach
GST0806	1.16	0.126	Small numbers diffusely embolized *Neospirorchis* eggs
GST0807	1.17	0.105	Small numbers diffusely embolized *Neospirorchis* eggs
GST0808	1.31	0.110	Small numbers diffusely embolized *Neospirorchis* eggs
GST0809	1.26	0.092	None present
GST0810	1.31	0.120	Small numbers diffusely embolized *Neospirorchis* eggs
GST0811	1.12	0.093	Small numbers diffusely embolized *Neospirorchis* eggs
GST0812	1.14	0.117	Small numbers diffusely embolized *Neospirorchis* eggs
GST0813	1.29	0.061	Small numbers diffusely embolized *Neospirorchis* eggs
GST0814	1.10	0.101	Adult *Neospirorchis* in thyroid gland
GST0815	1.15	0.128	*Neospirorchis* egg masses in thyroid gland
GST0816	1.17	0.107	Moderate numbers *Neospirorchis* eggs in thyroid; moderate embolization to embolized to lungs, stomach, intestine
GST0817	1.22	0.102	Small numbers diffusely embolized *Neospirorchis* eggs
GST0818	1.19	0.069	None present
GST0820	1.30	0.142	Moderate to large numbers *Neospirorchis* eggs and adults in thyroid gland; moderate embolization to lungs, stomach, intestine
GST0821	1.27	0.135	Adult *Neospirorchis* in thyroid gland
GST0822	1.12	0.088	Small numbers diffusely embolized *Neospirorchis* eggs; *Neospirorchis* egg masses in gastric submucosa
GST0823	1.18	0.121	Small numbers diffusely embolized *Neospirorchis* eggs
GST0824	1.17	0.058	Moderate numbers *Neospirorchis* eggs and adults in thyroid; moderate embolization to lungs, stomach, intestine
GST0825	1.13	0.112	Adult *Neospirorchis* in thyroid gland
GST0826	1.11	0.098	Small numbers diffusely embolized *Neospirorchis* eggs
GST0827	1.34	0.154	*Neospirorchis* egg masses in thyroid gland
GST0828	1.13	0.076	Large numbers *Neospirorchis* eggs in thyroid; moderate embolization to stomach/intestine
GST0830	1.30	0.072	Small numbers diffusely embolized *Neospirorchis* eggs
GST0831	1.13	0.094	Adult *Neospirorchis* in thyroid gland

CHAPTER 3
GENETIC DIVERSITY OF MARINE SPIRORCHIIDS AND CORRELATION WITH MORPHOLOGY, MICROHABITAT USAGE, AND HOST SPECIES

Introduction

Spirorchiids trematodes have long been recognized as parasites and causes of disease in sea turtles. Studies of the spirorchiids have been conducted for nearly 150 years and the list of described species and their turtle hosts has become extensive (Smith, 1997b). Taxonomy of these parasites, however, has been complicated by divergent morphology, inadequate specimen database, incompletely described species, and lack of accessibility to some type specimens (Platt, 2002). Furthermore, identification is problematic or impossible in many instances due the difficulty in obtaining suitable specimens. The genus *Neospirorchis* has been exceptionally difficult to study in Florida sea turtles because of its small size, elongate (filiform) body type, and tendency for adult parasites to inhabit small vessels. Also, the relatively delicate tegument of spirorchiids as compared to the connective tissue elements of host tissues limits the utility of enzymatic digestion techniques in harvesting parasites. Collected specimens often are fragmented, incomplete, or otherwise unsuitable for confident morphological examination. Inability to accurately identify spirorchiids not only limits our understanding of the taxonomy and biology of these parasites, but also their relationship, including associated disease, in their sea turtle hosts.

Molecular approaches as an adjunct to morphological and other methods of parasitological studies can be used to address problems created by incomplete or suboptimal specimens, phenotypic plasticity, questionable validity of some morphological features, and the potential time-lag between speciation and evolution of distinguishing morphological features (Nolan and Cribb, 2005). Most molecular studies of Digenea have examined ribosomal and/or

mitochondrial DNA, and of these targets the internal transcribed spacer (ITS) regions of the ribosomal gene and the mitochondrial cytochrome oxidase I (MCOI) gene have been the most extensively examined (Nolan and Cribb, 2005; Vilas et al., 2005). The ITS and MCOI have several desirable features for this purpose in that both are relatively easy to characterize, both exhibit a rapid evolutionary rate, and in many instances, both provide a clear indication of the presence or absence of genetic diversity that may be used in assessment of biological species diversity.

In the present study, a combination of molecular and classical parasitological approaches was used to prospect for and investigate the diversity of spirorchiids in sea turtles. Study objectives included: assessment of genetic diversity in some recognized species of spirorchiids, including specimens from Atlantic, Caribbean, and Pacific regions; genetic characterization of the two described species of *Neospirorchis* and comparison with *Neospirorchis* specimens collected from stranded sea turtles in Florida; and correlation of genetic data from *Neospirorchis* with microhabitat utilization, definitive host species, and available morphological features. Actual species delimitation was not feasible due to significant knowledge gaps in parasite life cycle, morphology, data from other geographical regions, and host range. The findings of this study, however, support that the diversity of marine spirorchiids far exceeds what has been previously recognized, especially for parasites tentatively classified within the genus *Neospirorchis*. Furthermore, the correlation of genetic findings with limited biological and morphological data has implications on both taxonomy and infection in the sea turtle host.

Methods and Materials

Parasite Specimens

Most of the parasites examined were recovered from dead stranded sea turtles in Florida from November 2004 through August 2008 that were recovered by the Sea Turtle Stranding and

Salvage Network (STSSN). Parasites were collected during complete necropsies using targeting collection techniques that included a combination of gross examination, examination of organs using a dissecting microscope, and examination of filtrates of blood, body fluids, washes of organs using a #45 sieve. For each parasite specimen, the host species, host identification number, and anatomic location were noted. Samples of eggs were placed into two categories, diffuse embolization or localized deposition. Localized deposition was recognized as the occurrence of distinct egg masses, which often displaced or effaced host tissue and frequently were associated with adult parasites, whereas, diffusely embolized eggs were observed as individual eggs without any apparent association with nearby adult spirorchiids. Spirorchiids with a filiform body type and/or ovoid, operculated eggs that lacked processes were tentatively identified as *Neospirorchis* species. Intact or nearly intact parasites were collected as morphological voucher specimens and were fixed in alcohol-formalin-acetic acid (AFA) and stained by routine methods. Representative specimens (including adults and eggs) were frozen at -80º Celsius. In addition, permission was granted to extract DNA from original voucher specimens of *N. schistosomatoides* submitted to the U.S. National Parasite Collection (Beltsville, Maryland) by TG Rand (accession 77971) (Rand and Wiles, 1985). Other parasite specimens included limited numbers of *Learedius learedi* and *Hapalotrema postrochis* collected from adult female *Chelonia mydas* in Tortugeuro National Park, Costa Rica by M. Santoro, *L. learedi* and *H. postorchis* collected by Hawaiian *C. mydas* by T. Work, and *L. learedi* obtained from captive *C. mydas* at Cayman Turtle Farm, Limited on Grand Cayman Island.

PCR and Sequencing

DNA was extracted from frozen or ethanol-fixed samples using the DNeasy Kit (Qiagen, Valencia, CA). Primer sequences for PCR and sequencing of the ITS2 were designed using comparative alignments from mammalian and avian schistosomes and two additional primers

described by Dvořák et al. (2002) were used to amplify the ITS1 (Figure 3-1). The MCOI was amplified use the forward primer JB3 – (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and the reverse primer COI-R – (5'-CACCAAATCATGATGCAAAAGG-3') (Miura et al, 2005). The reverse primer JB4.5 – (5' TAAAGAAAGAACATAATGAAAATG-3') also was used (Bowles et al, 1995). From the initial sequences, the custom forward primer MCOIF (5'-TGGGCATCCTGAGGTTTATG-3') and the custom reverse primer MCOIR (5'-TAAAGAAAGAACATAATGAAAATGA-3') were designed to avoid amplification of *C. caretta* MCOI. Standard PCR was performed using the Taqman PCR kit (Qiagen) in a 20μl reaction volume according to standard protocol. The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid, Franklin, MA). Reaction conditions for ITS amplification included initial denaturation at 95°C for 5 min, then 45 cycles of denaturation at 95°C for 60 s; annealing at 50°C for 45 s, and DNA extension at 72°C for 120 s, followed by a final extension step at 72°C for 10 min. Similar conditions were used for the MCOI primers, except for the annealing temperature, which was set at 45°C. The PCR products were resolved in 1% agarose gels. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, NJ) and analyzed on ABI 377 automated DNA sequencers at the University of Florida Center for Mammalian Genetics DNA Sequencing Facilities. All sequences were bidirectional with a minimum of approximately 75% overlap or were repeated to obtain consensus sequence. All chromatograms were manually reviewed using FinchTV 1.4.0 and any ambiguous or questionable data were discarded or repeated for confirmation. Nucleotide variation represented by single specimens was consistently re-evaluated by repeated PCR and sequencing and, in some instances, repeated DNA extraction from additional samples.

Sequence Alignments and Comparisons

All sequences were aligned and examined using Mega 4.0 (Kumar et al., 2008). Parasites initially were categorized by ITS2 sequence based on the findings of studies of other Digenea that support minimal or no intraspecific variation in the ITS. Comparisons of ITS1, MCOI, and biological data, when available, were then examined within and between genotypes for further evidence of similarity or diversity. Pairwise distances were calculated using Mega 4.0 based ClustalW alignments and default parameters with complete deletion of gaps and missing data. The predicted translation of the MCOI sequence was determined using the Virtual Ribosome (Wernersson, 2006) with the trematode mitochondrial translation. Where appropriate, differences in pairwise distances were tested for significance and evaluated graphically using the statistical program Analyze-it® Version 2.11.

Phylogenetic Analyses

Phylogenetic analyses were performed on ITS2 and MCOI sequences obtained from specimens of *Neospirorchis*. Homologous ITS2 sequences were aligned using ClustalW (Thompson et al., 1994), T-Coffee (Notredame et al., 2000), and MUSCLE (Edgar, 2004). No gaps were present in the predicted 142 amino acid sequence of the MCOI, thus alignments were performed using only ClustalW.

Bayesian analyses were conducted on each alignment using Mr.Bayes 3.1 (Huelsenbeck and Ronquist, 2001) using gamma distributed rate variation and a proportion of invariant sites. In addition, mixed amino acid substitution models were included in the analyses of MCOI sequences. The first ten percent of 1,000,000 iterations were discarded from the analyses as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989). Each alignment was run using

DNA Maximum Likelihood (DNAML) program for the ITS2 and the Protein Maximum Likelihood (ProML) program for the MCOI. For analysis of MCOI, three amino acid substitution models were compared, including JTT (Jones, Taylor et al. 1992), PMB (Veerassamy et al., 2003), and PAM (Kosiol and Goldman, 2005). Other set parameters included global rearrangements, five replications of random input order, and gamma plus invariant rate distributions. All analyses were unrooted. The values for the alpha of the gamma distributions and proportion of invariant sites were taken from the Bayesian analyses. Members of two other spirorchiid genera, *Hapalotrema mistroides* and *Learedius learedi*, were used as an outgroup. Data subsets were then generated from the ITS2 alignment that produced the most likely tree according to both Bayesian and maximum likelihood results and were analyzed using bootstrap analysis to evaluate the strength of the tree topology (200 re-samplings) (Felsenstein, 1985). For the MCOI, these analyses were performed on the most likely tree resulting from the three amino acid substitution models and bootstrap analysis was based on 100 re-samplings (greater than 100 re-samplings produced a fatal computer error).

Results

ITS2 and MCOI Sequencing

Hapalotrema species

Specimens from which sequence data were collected are listed in Table 3-1. Twenty four adult *Hapalotrema* specimens representing four different species were examined. Sequence data were collected from eggs from three additional specimens, but were not included in the genetic analysis. Pairwise distances between ITS2 and MCOI sequences obtained from *Hapalotrema* species, *L. learedi* and *C. bipora* are given in Tables 3-2 and 3-3.

The ITS2 was 100% homologous for all thirteen adult *H. mistroides* examined (Figure 3-2). Identical ITS2 sequence also was obtained from *Hapalotrema* eggs from two cases, one case

associated with polyp formation in the colon and another with egg masses in subserosal vessels. Slight variation in the MCOI nucleic acid sequence was observed (Figure 3-3). Five variants were identified and differed by only one or two nucleotides over 367 positions (>99% homology). Of the two turtles from which multiple *H. mistroides* were examined, both had two different variants represented. These minor differences in the nucleotide sequence did not result in any differences in the predicted amino acid translation among specimens of *H. mistroides* (Figure 3-4).

Specimens of *H. postorchis* included three localities: Hawaii, Florida, and the Caribbean (Costa Rica). Four base pair positions of 270 were different (98.5% homology) between the Hawaiian specimens and *H. postorchis* from Florida and the Caribbean (Figure 3-2). No mitochondrial sequence could be amplified from the Costa Rican samples, but the partial MCOI was sequenced for most Hawaiian and Floridian samples. Two slightly different variants in nucleotide sequence were identified among the Florida *H. postorchis* (Figure 3-3). One variant was represented by a single parasite and was different at one base position (>99% homology) that did not alter the predicted translation. The MCOI nucleic acid sequences for all Hawaiian *H. postorchis* were 100% homologous. Florida and Hawaiian MCOI nucleotide sequences were different at 26/367 nucleotide positions (92.9% homology), which resulted in three amino acid differences (97.5% homology) (Figure 3-4). The pairwise distances between the MCOI nucleic acid sequences of Florida and Hawaii parasites were significantly greater than between individuals of the same locality ($p<0.0001$).

All specimens identified as *H. pambanensis* had identical ITS2 sequences (Figure 3-2). Four variant partial MCOI sequences were amplified and were different at one or two of 367 base positions (>99% homology) (Figure 3-3). Multiple (3) variants were identified in one of

two turtles from which multiple parasites were examined. The predicted amino acid translation was 100% homologous for all specimens (Figure 3-4).

The ITS2 was 100% homologous for four specimens of the novel *Hapalotrema* species (Figure 3-2). Identical ITS2 sequence was obtained from embolized eggs from the infected animal. The eggs exhibited the typical bipolar processes observed in *Hapalotrema* species. One specimen had a single nucleotide difference in the MCOI sequence (Figure 3-3) that did not alter the predicted amino acid sequence (Figures 3-4).

Learedius learedi

The ITS2 was sequenced from a total of 45 individual *L. learedi* from four localities, including Hawaii, Florida, and the two Caribbean localities (Table 3-1). The ITS2 sequences were greater than 99% homologous and consisted of two variants, designated A and B, that had two nucleotide differences over the 299 positions examined (Figure 3-5). Variant A included some Florida *L. learedi*, all CTFL parasites, and the one TNP sample. The B variant was found in all of the Hawaiian *L. learedi*, as well as parasites from two Florida *C. mydas*. Both Florida turtles with the variant B also were infected by parasites with the variant A. Greater diversity was detected in the MCOI nucleic acid sequences, which included thirteen individual variants with as many as thirty-five nucleic acid differences over the 418 positions examined (91.6% homology) (Figure 3-6). In contrast to the ITS2 sequence, representatives of each MCOI variant were limited to one locality. Predicted translation of the MCOI resulted in only two variants, designated A and B, that differed by two amino acids (98.6% homology) (Figure 3-7). Variant B was represented by three Hawaiian *L. learedi*, and all remaining specimens were variant A. Pairwise distances in MCOI nucleotide sequence were compared between Hawaiian and Floridian *L. learedi* and between ITS2 variants (Table 3-3, Figure 3-8). The median distance between individuals from different localities was significantly greater than between individuals

of the same locality (p<0.0001). Also, there was significantly more variation between individuals with different ITS2 sequences as compared to those with homologous sequence (p<0.0001). There was, however, significant overlap in the ranges of pairwise distances observed between and within localities and ITS2 genotypes. When both ITS2 variant, locality, and MCOI variant were considered, the Florida ITS variant B and Hawaiian MCOI variant A were most similar (Figure 3-8).

Carettacola bipora

The ITS2 was sequenced from five *C. bipora* from three different *C. caretta* (Table 3-1) and was found to be identical (Figure 3-9). Additionally, the partial MCOI was sequenced from three parasites and all were 100% homologous (Figure 3-10).

***Neospirorchis* species**

Adults or eggs from 234 examples of *Neospirorchis* species were collected from a total of 91 stranded turtles, including 60 *C. caretta*, 28 *C. mydas*, and three *L. kempi* (Table 3-4). Confident morphological identification was only possible in two circumstances. The first was original voucher specimens of *N. schistosomatoides* from the U.S. National Parasite Collection (Rand and Wiles, 1985). DNA was successfully extracted and amplified from two fragmented specimens. Second, several examples of *N. pricei* were observed in Florida *C. caretta* and were collected intact in condition suitable for morphological identification.

The complete ITS2 and partial MCOI gene were sequenced for most specimens. The lengths of ITS2 sequences ranged from 271 to 298 base pairs, which did not include fifteen base pairs from the 5'end that were part of the forward primer (Figure 3-11). An overlapping 429 partial nucleotide sequence of the MCOI was obtained for most specimens (Figure 3-12) with a predicted translation of 142 amino acids (Figure 3-13). Nineteen different genotypes were identified in which there was minimal or no variation between examples in either the ITS2

and/or MCOI. As in the other examined spirorchiid genera, the nucleic acid sequence of the MCOI was much more variable than the ITS2. Much of this variation did not alter the amino acid sequence of the MCOI, which yielded distances similar to the ITS2 in pairwise comparisons. Parasites in which only partial sequence data were collected included Neogen-15 (incomplete ITS2, complete MCOI), Neogen-16 (ITS2 data only), and Neogen-19 (MCOI data only). In addition, shorter MCOI sequences were obtained for Neogen-17, 18, and 19.

The ITS1 also was completely or partially sequenced for some examples (data not shown); however, many parasites had evidence of intragenomic variation due to variable numbers of internal repeats. Amplification of the ITS1 often yielded consistent patterns of products within genotypes when bands were visualized by electrophoresis (Figure 3-14). Some ITS1 versions were greater than 1,500 base pairs in length. This variation confounded sequencing efforts, alignments, and comparisons in many instances because selection of the appropriate "variant" could not be confidently determined. Thus, classification by genotype and phylogenetic analyses were based on the ITS2 and MCOI data.

Phylogenetic Analysis

For the ITS2, Bayesian phylogenetic analysis using the T-Coffee alignment resulted in a greater harmonic mean of estimated marginal likelihoods as compared to ClustalW and MUSCLE alignments. The branching patterns were not significantly different. The Bayesian tree based on ITS2 sequence using the T-Coffee alignment is shown in Figure 3-15. The Mtmam model of amino acid substitution for the MCOI sequence was found to be the most probable with a posterior probability of 1.00 (Cao et al., 1998; Yang, Nielsen, and Hasegawa, 1998). The tree resulting from Bayesian analysis using the ClustalW alignments of the MCOI amino acid sequence is given in Figure 3-16.

The most likely trees were determined from the ITS2 alignment (T-Coffee) and MCOI alignment (ClustalW) by ML analysis and these parameters were applied to the bootstrap analyses. The JTT model of amino acid substitution was found to be the most likely for the MCOI sequence. As expected, the ML analyses produced a less resolved tree as compared the Bayesian analyses. The bootstrap values from ML analyses are shown on the ITS2 (Figure 3-15) and MCOI Bayesian trees (Figure 3-16).

The ITS2 and MCOI produced similar trees with good support for a clade that included all *Neospirorchis* collected from the gastrointestinal tract (Neogen-9 through 14). Support for this clade was weakest in the ML analysis of the MCOI sequences. Both trees also demonstrated good support for grouping to two similar *Neospirochis* types (based on limited morphology and egg size) represented by Neogen-9 and 11. Analysis of the ITS2 also support grouping of Neogen-13 and 14, which were both amplified from distinctive eggs with surface projections, as will be discussed subsequently, which were not observed in other specimens. This grouping was not indicated in the MCOI tree. The distances estimated between the gastrointestinal *Neospirorchis* and other forms were comparable to that observed between the genera *Hapalotrema* and *Learedius*. There was poor resolution of the phylogeny of non-gastrointestinal *Neospirorchis*, with very weak support of an inclusive clade in the MCOI analysis. Further consideration of phylogenetic results is given under discussion of individual genotypes.

Genotypes by Microhabitat and Host Species

The anatomic locations or microhabitats from which spirorchiids were collected and the results of genetic analyses revealed many consistent relationships. Specific host microhabitats often were associated with genotypes represented by adult spirorchiids and/or localized egg deposition (Table 3-5). Furthermore, most genotypes were only found in one host species. The general categories of microhabitats observed were the submucosal vasculature of the

gastrointestinal tract (GI) and vessels of non-gastrointestinal organs (NGI). Sites included under NGI included the central nervous system, endocrine organs, gonads, and heart. The degree of similarity among genotypes recovered from like microhabitats observed in the phylogenetic analyses were reflected in lower p-distances in both the ITS2 and MCOI (Table 3-6). In the following discussion, groups of genotypes recovered from similar microhabitats will be presented and compared by apparent organ distribution.

Neogen-1, 2, and 3, were found exclusively in the leptomeninges of the CNS. These three genotypes were found in the 40 of 43 instances in which adult spirorchiids or locally deposited egg masses were removed from the CNS, and were not identified in parasites from other sites. These spirorchiids were observed as elongate, filiform adults and/or discrete egg masses within leptomeningeal vessels, as is typical of neurospirorchiidiasis in sea turtles. Neogen-1 corresponded to *N. schistosomatoides*, as supported by genetic characterization of two individual voucher specimens, and was observed in 20 *C. caretta* and one *L. kempi*. Anterior and posterior fragments from Florida parasites that were identified with the Neogen-1 genotype were compatible with *N. schistosomatoides* based on limited examination. Neogen-2 was restricted to *C. caretta* (n=14 turtles) and Neogen-3 was only found in *C. mydas* (n=4 turtles). The three examples of other genotypes identified in the central nervous system included two cases in which adult *Neospirorchis* of other NGI genotypes (Neogen-5 and 6) were recovered and one case where a single egg mass was examined (Neogen-18), but no adults were observed. There was no variation in either ITS2 or MCOI within the Neogen-1 and Neogen-2 genotypes. Neogen-3 included one ITS2 variant (B) that had two substitutions across 302 positions (>99% homology) as compared to the other three representatives (variant A) of this genotype. This difference corresponded to some variability in the MCOI nucleic acid sequence. Of the 429 nucleotide

sequence of the MCOI examined, the parasite with variant B ITS2 had 14 nucleic acid differences (96.7% homology) as compared to variant A examples, which were identical. The predicted MCOI amino acid sequence for Neogen-3, however, was identical for all examples. In pairwise comparisons between the three genotypes (Table 3-6), there were between fourteen and sixteen base pair differences (94.7 to 95.4% homology) in the ITS2 and between 32 and 38 differences in the MCOI (90.9 to 92.3% homology).

Four genotypes, Neogen-4, 5, 6, and 7, primarily were found in parasites collected from the vessels of endocrine organs, especially the thyroid gland and adrenal glands, and thymus (Table 3-5). A few examples also were recovered from the liver and testis. These spirorchiids were most often observed either as single or a few adult spirorchiids, discrete egg masses, or as infections with many adults and/or eggs resulting in distention and obstruction of vessels and effacement of the host tissue. No other genotypes were detected among the 40 adult spirorchiids collected from endocrine organs, thymus, or gonad. Furthermore, only one other genotype, Neogen-16, was observed in one of 38 genetic characterizations of eggs recovered from these sites. In four cases in which parasitism of the pineal gland was observed, Neogen-4 or 7 were identified in adults or egg masses in all specimens. Notably, no other genotypes, including those frequently found in the leptomeninges, were recovered from the pineal gland. Neogen-6 was characterized in the least number of samples and included only three adult specimens, which were recovered from very different anatomic locations. Two adult spirorchiids were flushed from hepatic vessels of one turtle and in another case, adults were found migrating within the optic nerves, suggesting that adults with the Neogen-6 may be relatively widespread. However, egg masses within thyroid and thymic vessels, as was frequently observed in parasites with genotypes 4 and 5, also were identified with the Neogen-6 genotype in one case. The only other

example of characterization of one of these genotypes outside the sites previously listed (other than diffusely embolized eggs) was one case in which Neogen-5 was amplified from a single adult collected from the leptomeninges of a *C. caretta*. Neogen-4 and 6 were recovered exclusively from *C. caretta* and Neogen-7 was only found in *C. mydas*. Neogen-5 was found in both *C. caretta* and *L. kempi*. The ITS2 and MCOI sequences were identical for all specimens of Neogen-4 (n=46), Neogen-5 (n=14), and Neogen-7 (n=36). Slight variation in the ITS2 was observed in two examples of Neogen-6 from the same individual host and consisted of a single substitution across the ITS2 (302 base pair sequence). Unfortunately, repeated attempts to sequence the MCOI from this material were unsuccessful; however, this variant was 100% homologous with another Neogen-6 specimen across a 444 base pair portion of the ITS1, which included the 3' region. Pairwise distances between genotypes are given in Table 3-6. There were between 11 and 16 nucleotide differences between ITS2 sequences (94.9% to 96.4% homology) of the different genotypes that corresponded to 24 to 36 nucleotide differences in the MCOI sequence (94.2% to 91.3% homology).

Neogen-8 was consistently detected in nine specimens identified as *N. pricei*. Adult *N. pricei* primarily were recovered from the cardiac chambers and major vessels, which is consistent with the original description and only report of this parasite (Manter and Larson, 1950). All examples were found in *C. caretta*. The ITS2 was identical in all specimens and only single nucleotide differences in the MCOI were observed in the two adult specimens. There was no alteration of the predicted amino acid sequence.

The next several genotypes, Neogen-9, 10, 11, 12, 13, and 14, were obtained from adults found within the gastrointestinal submucosa associated with localized egg deposition. Examples with genotypes 9, 10, 11, and 12 were observed grossly as black, serpiginous egg masses in the

alimentary submucosa often with an associated adult that tended to course extensively through submucosal vessels. Some distinctive features were present in parasites of some genotypes; however, adequate specimens could not be obtained for detailed morphological examination. Neogen-10 was consistently amplified from adult parasites noted to have a distinct yellow color and relatively large egg size (average of 59 x 47 μm). Neogen-9 and 11 were detected from adults that were very similar in appearance and were characterized by white, very delicate adults and smaller egg size (average of 43 x 36 μm) as compared to Neogen-10. Only one adult parasite was identified with the Neogen-12 genotype and the remaining examples were obtained from egg masses. In contrast to these four genotypes, Neogen-13 and 14 appeared to have a more restricted distribution and were found as small discrete egg masses in the stomach (both) or colon (Neogen-14 only). The eggs of both genotypes had distinctive surface processes visible by light and electron microscopy. Neogen-10, 11, and 12 were recovered from *C. caretta* and Neogen-14 was only identified in *C. mydas*. Two alimentary forms were identified in multiple hosts including Neogen-9, which was recovered from both *C. caretta* and *L. kempi*, and Neogen-13, which was found in both *C. caretta* and *C. mydas*. Neogen-13 was the only genotype found in both *C. caretta* and *C. mydas* in this study. All individuals within each GI genotype had identical ITS2 sequences. Individuals of three genotypes, Neogen-10 (n=13), Neogen-11 (n=11), and Neogen-12 (n=4), also had identical MCOI sequences. One or two nucleotide differences were observed in the MCOI sequences of Neogen-9 (n=15), Neogen-13 (n=4), and Neogen-14 (n=4). The only resulting variation in the predicted amino acid sequence was for Neogen-14, which was represented by two variants with two amino acid differences. Pairwise distances between genotypes are shown in Table 3-6. Neogen-9 and 11 were the most similar with only 4 nucleotide differences (98.7% homology) in the ITS2 and 16 to 17 nucleotide differences in the

MCOI (95.9% to 96.2% homology) that translated into one predicted amino acid difference. Neogen-13 and 14 were the next most similar, with 14 differences (95.3% homology) in the ITS2 sequence, 42 to 46 differences (87.2% to 88.4% homology) in MCOI nucleotide sequence and ten and eleven differences in predicted amino acid sequence (92.3% to 93.0% homology). The remaining GI genotypes had between 31 and 51 nucleotide differences (83.1% to 89.6% homology) in ITS2 sequences and relatively similar differences in the MCOI sequences that ranged from 44 to 69 nucleotide differences (83.4% to 96.1% homology) and seven to 19 amino acid differences (86.6% to 95.1% homology).

The remaining genotypes were amplified from eggs and no examples were associated with adult parasites. Also, most of these genotypes were found in only a few cases. The most distinctive distribution of eggs was observed in specimens identified with Neogen-15 (n=5), which was amplified from small discrete egg masses in the cloaca and urinary bladder of *C. caretta*. This genotype was most similar to the GI forms based on available ITS2 sequence, which included a short sequence of 173 nucleotides. Attempts to sequence the remaining 3' fraction of the ITS2 were unsuccessful, thus this genotype was not included in the ITS2 phylogenetic analysis. The MCOI, however, was successfully sequenced and had equivalent distances to both the GI and NGI forms, as reflected in its exclusion from the GI clade in the MCOI phylogenetic analysis. With the exception of Neogen-17 (n=1), which was found in a small egg mass in the leptomeninges of a single *C. mydas*, the remaining genotypes were detected in diffusely embolized eggs. Neogen-16 (n=1), 18 (n=4), and 19 (n=2) were only found in *C. mydas*. Both Neogen-18 and 19 were found in eggs from multiple sites in infected turtles. The p-distance data and phylogenetic analysis support that the genotypes Neogen16 through 19 are most similar to NGI forms (Table 3-6).

Mixed Infections and Embolized Egg Data

Parasites from multiple sites were collected and sequenced from 62 turtles. Of these, eighteen had only one genotype, twenty-three had two genotypes, eleven had three genotypes, and ten had four or five genotypes. Multiple parasite samples, either multiple adults, adults and eggs, or multiple egg samples, from the same specific site (not including different areas within the intestine) were examined in six instances, and concordant results were obtained in five. The discordant result was sequence obtained from egg masses from the leptomeninges of an infected loggerhead that yielded mixed results and evidence of three different genotypes. Mixed sequence data were observed in only two other examples, and in both cases the source materials also were egg masses, which are easily comprised of eggs from multiple sources. Despite this risk of "mixed" samples in DNA extractions from egg masses, unambiguous sequence data were collected from 117 of 120 examples of source materials containing eggs.

Mixed infections by genotypes with similar apparent tropisms were documented in some cases. Most commonly observed was recovery of multiple different genotypes associated with adult *Neospirorchis* and or egg masses in the gastrointestinal tract. Of the eleven turtles from which multiple samples were collected from the submucosa, multiple genotypes were detected in seven. In most instances, it was elected to sequence multiple parasites because the adults had a dissimilar sub-gross appearance, e.g. yellow or white coloration, and/or different egg sizes were observed. Infection by multiple genotypes with a tropism for the endocrine organs and non-gastrointestinal organs, including Neogen-4 through 7, was less frequently observed. Individual genotypes were detected from multiple sites, typically either the thyroid and thymus or multiple other endocrine organs, in twenty-eight cases. Mixed infections were detected in only four turtles. One turtle had mixed sequence including both Neogen-4 and 5 in the thyroid gland. Another turtle had embolized eggs in the lung from which Neogen 6 was amplified and Neogen-

4 was detected from three other sites. A third example had Neogen-4 amplified from adult *Neospirorchis* from the thyroid gland, thymus, testis and adrenal gland, whereas two adult *Neospirorchis* flushed from the liver had the Neogen-6 genotype. In the fourth example, Neogen-5 was detected in adults from the thyroid gland and testis and Neogen-4 was amplified from a single adult in the leptomeninges.

Eggs that were diffusely embolized to organs were examined in 26 turtles. Twenty two were characterized as spirorchiids with endocrine organ microhabitats (Neogen-4, 5, 6 or 7), two were Neogen-8 (*N. pricei*), two were Neogen-18, and one was Neogen-19. In half of these cases, all of which infected with spirorchiids of the genotypes Neogen-4, 5, 6, or 7, adult *Neospirorchis* sp. or localized egg deposition with the same genotype was identified in endocrine organs. Consistency of genotype between the spirorchiid adults recovered and eggs found in distant sites was most readily observed in a group of *C. mydas* that died during a hypothermic stunning event. Seven turtles had adult *Neospirorchis* with the genotype Neogen-7 in the thyroid gland, and all had eggs of the same size and with same genotype detected in a diffusely embolized pattern in one or more organs, including the lungs, gastric mucosa, and/or enteric mucosa. Only four cases had discordant results in which a genotype with a similar organ tropism was detected, but the embolized eggs yielded a different genotype. Congruence of results could not be determined in nine additional cases. In these cases, either a single specimen was examined or embolized eggs represented the genotype, i.e. the location of adult parasites is unknown (Neogen-16 through 19).

Discussion

Application of Genetic Findings and Implications

In a review of the use of ribosomal DNA in studies of Digenea, Nolan and Cribb (2005) recommended several guidelines for effective molecular approaches to trematode studies. Efforts were made to incorporate these guidelines to whatever extent was possible. Specifically,

relatively large numbers of individual parasites were examined and a broad range of specimens was sought to include three host species and any parasites that appeared to exhibit distinct morphological features or use of different microhabitats. Also, specimens of some species were kindly provided from other regions. Replicates were found for many of the discovered genotypes and all genetic sequences were carefully scrutinized and differences were investigated by repeated PCR and sequencing. In addition, two different genetic markers were used to investigate results. Furthermore, as discussed below, the genetic results were evaluated in the context of available biological and morphological data. The limitations of this study were largely due to the relative restrictions of working with protected, ocean-going species and the problematic morphological features of the genus *Neospirorchis*. Limitations that must be considered included the limited understanding of spirorchiids in terms of morphology (especially *Neospirorchis*), host range, life cycle, and geographic distribution, as well as the inability to collect adequate voucher specimens for *Neospirorchis*. With exception of one *Hapalotrema* species, the data collected during the study do not meet the classical requirement for species designation, which is morphological examination of sexually mature adult trematodes. Thus, new species were not proposed nor were there attempts to delimit individual species within the genetic data. Rather, these findings are presented as evidence of previously unrecognized diversity in marine spirorchiids with the following relevant applications: 1) a basis for hypotheses regarding regional differences and biological species diversity; 2) a comparative genetic reference for other spirorchiid species and parasites from other geographic regions; 3) an alternative means of species identification for partial specimens, eggs, or immature forms; and 4) an important consideration in disease studies, parasitological surveys, and clinical diagnosis.

Comparison of Genetic Diversity with Other Studies of Digenea

Although it is not proposed to partition the genetic data from this study into species or to divide recognized species, there is ample support for the presence of clearly distinct forms, as well as more subtle differences that require further examination. Comparison of genetic variation across taxa, referred to as the "genetic yardstick" approach, is not appropriate for delimiting species, but is useful for formulating hypotheses and identifying parasites that may possess unrecognized or cryptic differences (Vilas et al., 2005). In the following discussion, results of genetic characterization of the various marine spirorchiids will be compared with observations in other digenean trematodes with an emphasis on significance of genetic diversity.

The ITS is one of the most commonly used genetic markers in trematode studies and is useful because limited or no variation has been observed within species, with few exceptions. In a review of 63 studies that incorporated the use of the ITS to study digenean trematodes, almost all examples of intraspecific variation were suspected to be due to error, either in sample identification or labeling, or failure to recognize multiple species, as was clearly evidenced in multiple examples (Nolan and Cribb, 2005). Another potential source of erroneous interpretation is variable numbers of tandem repeats in the ITS1, as was observed in multiple *Neospirorchis* genotypes in the current study. These data were largely omitted from analysis due to the uncertainly that appropriate variants were being compared. Some actual intraspecific differences may occur due to geographical variation, which has been investigated in Schistosomatidae, Fasciolidae, and Paragonimidae (reviewed by Nolan and Cribb, 2005). Blair et al. (2005) recommended subspecific status under a *Paragonimus skrjabini* species complex for several recognized species based on geographic origin, morphological, and molecular findings. Most studies, however, have found no variation from distant localities and fewer have found variation

that was interpreted as evidence of multiple species based on host usage and/or egg morphology (Nolan and Cribb, 2005).

Intraspecific variation is greater within the mitochondrial genes nicotinamide adenine dinucleotide dehydrogenase subunit I (NDI) and MCOI (Vilas et al., 2005). In a study of four different genera and seven species of digenea, maximum intraspecific differences observed were less than or equal to 2.3% in the NDI, which is reported to exhibit more divergence than the MCOI. It is suggested that distances of greater than 5% within mitochondrial sequences of specimens from the same population should be further investigated (Vilas et al., 2005).

Diversity of Spirorchiids of Sea turtles in Florida

Hapalotrema*, *Learedius*, and *Carettacola

No variation in the ITS2 and minimal or no variation MCOI was found in four of the spirorchiid species examined, including *H. mistroides*, *H. pambanensis*, a novel *Hapalotrema* species, and *C. bipora,* in which identification was supported by confident morphological study. *Hapalotrema mistroides*, *H. pambanensis* and *C. bipora* were represented by parasites from multiple turtle hosts that stranded in different areas of the Atlantic and Gulf Coasts of Florida. The apparent variation in organization of the testes of *H. pambanensis* was not reflected in any observed genetic difference, thus this observation is regarded as either artifact or phenotypic variation. Genetic differences were observed, however, in the two species in which specimens from distant localities were examined.

Multiple morphological voucher specimens from Hawaii, Florida, and Costa Rica were identified as *H. postorchis*. The ITS2 was identical within localities of *H. postorchis*, but minor differences of four nucleotides (98.5% homology) were observed between Hawaiian and Atlantic-Caribbean samples. Greater variation (93.0% homology) was observed in the MCOI nucleotide sequence between regions. Two possible explanations for these findings are that

differences reflect the presence of two different species or geographical variation. The pairwise distances of both the ITS2 and MCOI were comparable to that observed between different species of *Schistosoma* and *Paragonimus* (Vilas et al., 2005). Either explanation would not be surprising given that Atlantic-Mediterranean and Indian-Pacific *C. mydas* are estimated to have been isolated for as long as 1.5 to 3.0 million years (Bowen et al., 1992). Detailed correlative morphological studies of *H. postorchis* from both regions are necessary to investigate these different possibilities. However, assessment of other important biological characteristics, such as morphology of immature stages and intermediate host usage, will be limited until the life cycle is discovered.

Similar results were obtained from Atlantic and Pacific *L. learedi*, although the emergent picture was somewhat more complicated. Two ITS2 variants that differed by two nucleic acid positions were found in parasites identified as *L. learedi*, but did not correspond to the region of origin for the turtle host. Both variants were found in Florida *C. mydas*, and in both examples these turtles were co-infected by *Learedius* with the two different variants. The ITS2 of variant B was 100% homologous to that obtained from all Hawaiian *L. learedi*, which were identical. The MCIO data were informative in segregating specimens identified as *L. learedi* in Florida turtles. Individuals with different ITS2 variants had much greater pairwise distances (6.9% to 7.4%) as compared to individuals with like ITS2 sequences (less than 1%). This degree of difference in the MCOI is greater than the intraspecific variation reported in other digeneans from the same population (Vilas et al., 2005). In the case of *L. learedi* in the present study, the different genotypes actually were collected from the same individual host in two instances. Thus, there is evidence that *L. learedi* in Florida turtles may actually be two closely related species. Interestingly, one of the turtles from which both variants were identified was noted to

have very two different sizes of eggs on fecal examination that were both consistent in morphology with *Learedius* or *Hapalotrema* species. No *Hapalotrema* were recovered from this animal. No obvious differences were seen in the voucher specimens; however, some vouchers had been previously frozen and were not ideal for examination. More detailed study is necessary.

Variability also was observed MCOI sequences obtained from Hawaiian *L. learedi*. Pairwise distances as high as 5.7% were observed between samples and resulted in two amino acid differences in three individual parasites. Hawaiian sequences could be segregated into two groups based on the predicted amino acid sequence of the MCOI with some overlap in pairwise distances. This degree of variability within Hawaiian samples was comparable to that observed between *H. postorchis* from the Atlantic and Pacific and is higher than observed within other digenean species from the same populations (Vilas et al., 2005). Although the ITS2 was identical for Hawaiian *L. learedi*, the ITS2 may not be the most sensitive indicator of species differences, especially cryptic species (Nolan and Cribb, 2005). Examination of more *L. learedi* from additional *C. mydas* from Hawaii and other Pacific localities clearly is needed.

When Florida and Hawaiian genotypes of *L.learedi* were compared, Florida parasites with the variant B were more similar to the Hawaiian variant A than either genotype was to the other variant from the same region. The Florida variant A and Hawaii variant B had identical ITS2 sequences and very similar MCOI sequences (2.4% to 4.5% homology). This degree of variation in the MCOI was comparable to that observed within species of other trematode genera (Villas et al., 2005, Blair et al., 2005). We hypothesize that the *Learedius* represented by the Florida A variant and Hawaiian B variant is the same species, although there is evidence of a much more complicated population structure in both regions that requires further investigation.

***Neospirorchis* species**

The genetic data obtained from *Neospirorchis* sp. support that the diversity of this genus is far greater than previously recognized and members fall into two general groups, those with an apparent tropism for the vasculature of the gastrointestinal tract and those found in vessels of non-gastrointestinal sites, including the nervous system and endocrine organs. Phylogenetic analyses support that the gastrointestinal group comprise a well-supported monophyletic clade that is different from other *Neospirorchis*, as indicated by the observed branch lengths. These differences are greater than that observed between the genera *Hapalotrema* and *Learedius*, suggesting that gastrointestinal forms may actually belong to a different genus. The remaining non-gastrointestinal forms, i.e. those in which adults and/or localized egg deposition was observed outside of the gastrointestinal tract or eggs were diffusely embolized, are more closely related and there was little statistical support for additional phylogenetic structure based on ITS2 and MCOI sequences.

The observations in Nolan and Cribb's extensive review of studies involving the ITS in digenean trematodes support that any differences in the ITS should be taken as an indicator of potential species diversity because current evidence of intraspecific variation was either suspect or, in the case of geographical variation, rarely observed. Considering the ITS data alone in the present study, fourteen distinct homologous genotypes were identified (Neogen-1,2,4,5,7,8,9,10,11,12,13,14,15,18) that were represented by multiple parasite specimens. Of those parasites that belonged within these fourteen distinct ITS2 genotypes that were represented by multiple examples, eight (Neogen-1,2,4,7,10,11,12,18) of the fourteen had identical MCOI sequences. Thus, eight of the *Neospirorchis* genotypes represented by multiple specimens had 100% homologous ITS2 and MCOI sequences. Less than 2% variation in the MCOI nucleic acid was observed in the remaining six ITS2 genotypes (Neogen-5,8,9,13,14,15), four of which only

had one or two nucleotide differences. This level of variation is less than the intraspecific differences documented in other digeneans (Vilas et al., 2005). Furthermore, resulting variation in the predicted amino acid sequence was only observed in Neogen-14. Therefore, fourteen of the ninteen *Neospirorchis* genotypes identified in this study had identical ITS2 sequences and exhibited either no variation or minimal variation in the mitochondrial sequence.

Additional studies are needed of the remaining five observed genotypes (Neogen-3,6,16,17,19). Slight variation in the ITS2 was observed in one of four examples of Neogen-3 and one of ten examples of Neogen-6. The variant observed within the Neogen-3, which was different at 2/302 positions in the ITS2, also was different at 14/429 nucleic acid positions (3.3%) in the MCOI sequence that did not result in any amino acid differences. Our interpretation is that it is premature to include the single Neogen-3 variant with the other three examples given the many unknown characteristics of these parasites and limited examination of only a small number of infected *C. mydas*. Careful examination of more meningeal *Neospirorchis* from *C. mydas* is needed. All four examples of Neogen-3 were collected from adult *C. mydas*, a nesting female in the case of the observed variant, which migrate long distances from foraging grounds to nesting beaches. It is possible that the observed variation reflects some degree of geographical variation. With regard to the variant in the Neogen-6, which had a single nucleic acid difference over 302 positions in the ITS2, this specimen was 100% homologous across 445 base pairs of the ITS1 as compared with other examples. Unfortunately, the MCOI sequence could not be amplified from the example with the variant, thus corroborative data from a second genetic marker were not available. This genotype was represented by very few adult parasites, which were found in diverse locations, including the liver and the perineurium of the optic nerve. Thus, Neogen-6 requires further characterization in

multiple respects, as do those genotypes that were identified from embolized eggs only or in which adults have not been collected (Neogen-15,16,17,18,19). Two distinct ITS2 genotypes (Neogen-16,17) were observed in single examples, both of which were from *C. mydas*, thus it cannot be stated whether or not the ITS2 is truly conserved; furthermore, the MCOI could not be amplified from Neogen-16. The MCOI sequence of Neogen-17; however, had distances ranging from 6.2% to 18.1% from other *Neospirorchis*, supporting that it is different. Lastly, the ITS2 could not be amplified from Neogen-19, which is represented by identical MCOI sequences from embolized eggs (2 different sites) from a *C. mydas*. As with Neogen-17, the distances observed, 7.2% to 19.6%, also suggest that this spirorchiid is a distinct form.

The next point of discussion is comparison of distances observed between genotypes and the evidence for biological or morphological differences between similar genotypes. It was previously mentioned that any differences in the ITS sequences between individuals warrant further investigation. Regarding the MCOI, one study that examined variation in mitochondrial sequences within and between species of multiple digenean genera suggested the rough guideline of distances greater than 5% as an indicator that multiple species may be present. In the present study, pairwise distances in MCOI sequence were below this mark in two examples, Neogen-3 and 5, and Neogen-9 and 11. In the case of Neogen-3 and 5, representatives with this genotype displayed differences in both the microhabitat and host species from which they were collected. Neogen-3 was only found in the leptomeninges of *C. mydas* (n=4), whereas Neogen-5 was found primarily in endocrine organs (n=13) and, in one instance, the leptomeninges and only in *C. caretta*. Furthermore, Neogen-3 was never detected in the 41 instances in which parasites were sequenced from the leptomeninges of *C. caretta* and Neogen-5 was not identified from any of the seventeen specimens obtained from the endocrine organs of *C. mydas*. These findings support

biological differences between parasites with relatively little associated genetic distance in MCOI sequences (4.5%). Notably, although pairwise distances between MCOI sequences were relatively low, there was no support for phylogenetic grouping of these genotypes in either the ITS2 or MCOI analyses. No such detectable biological differences were observed between Neogen-9 and 11, which formed a well-supported cluster in phylogenetic analyses of both genetic targets. There were four nucleotide differences in the ITS2 and 16 to 17 nucleotide differences in the MCOI between these genotypes. Spirorchiids with both genotypes were found in the enteric submucosa, the adults exhibited the same slender, white morphology, eggs were indistinguishable, and both were found in *C. caretta* in comparable numbers. Stranded turtles from which parasites were collected were from multiple zones on the Atlantic and Gulf coasts and in one instance both genotypes were found in the same turtle. Further examination of these apparently highly similar forms will require additional morphological and biological data.

Genotypes and Microhabitat

The association between spirorchiid genotype and select distribution in host organs, i.e. microhabitat use, was a remarkable finding in the present study. A lack of site fidelity has been noted in *Spirorchis* species and has been reported most often in the spirorchiid literature (Holliman, 1971; Platt, 1993; Goodchild and Kirk, 1960), although tropism for gastroenteric arterioles was described for *Spirorchis elephantis* and the heart and major vessels for *Vasotrema robustum* in early studies (Wall, 1941; Wall, 1951). Selective microhabitat use, however, is well-recognized for many blood flukes. For example, adults of both *Schistosoma mansoni* and *S. japonicum* migrate to mesenteric veins to mate and *S. haematobium* traffics to the vessels of the urinary bladder (Marquardt et al., 2000). Also, the bird parasite *Trichobilharzia regenti* migrates within nervous tissue to reach the nasal cavity (Blazová and Horák, 2005). Similarly, there was a strong association between most of the genotypes observed in this study and intrahost

distribution. One notable exception that conflictes with previous reports was *N. schistosomatoides*. The publication corresponding to the voucher specimen (Rand and Wiles, 1985) describes adults within the heart and the original description (Price, 1934) was from "visceral blood vessels," whereas, the associated genotype, Neogen-1, was consistently found in the CNS in Florida *C. caretta*. It is possible that the infections encountered by others were early and the parasites ultimately would have migrated to meningeal vessels. Furthermore, these specimens were collected from a *C. mydas*, whereas all Neogen-1 examples in the present study were found in *C. caretta*, thus species differences in host-parasite interaction are another consideration.

These apparent tropisms are relevant to the biology of the parasite, as well as the implications on the health of the host. Propagation strategy is not readily apparent in some examples where sites of intense egg deposition are seemingly remote from access to the external environment, such as in spirorchiids found within endocrine organs and the central nervous system. One group of turtles included in this study provided some insight. Several immature *C. mydas* that died from hypothermic stunning were found to be infected by spirorchiids of the Neogen-7 genotype that has only been observed this far in the thyroid gland and pineal gland. Adults and eggs were observed in the thyroid gland in these turtles; however, the most notable finding was large numbers of embolized eggs within the gastroenteric submucosa and pulmonary vessels. The eggs were all of comparable size and genetic analysis demonstrated that they were the same genotype as the adults found in the thyroid gland. The results provided a clearer indication of embolization patterns than was observed in *C. caretta* or older *C. mydas*, which were often found to be infected by multiple spirorchiid types. It appears that Neogen-7 very effectively disseminates eggs to sites where they may be expectorated and/or passed out in the

feces. The thyroscapular vein drains thyroid gland and surrounding region, flows into the precava, and empties into the sinus venosus (Wyneken, 2001). By this route, widespread egg embolization may occur. A similar strategy may be used by the CNS forms, although the vascular pathway is not as direct or in as close proximity to central venous flow. It also was considered that adults may migrate and oviposit within the nasal submucosa, as observed in *T. regenti* in waterfowl and *S. nasalis* in cattle; however, neither adults nor abundant eggs have been recovered from the nasal mucosa in turtles with neurospirorchiidiasis. The propogation strategy for the GI forms is more obvious and seemingly the most direct. Based on observations in necropsied turtles and genetic analysis of embolized eggs, most of the eggs produced by the GI forms appear to be locally deposited. One form represented by Neogen-15 has a similar egg distribution to *S. haematobium*. The adults correlating to these eggs have not been identified, although based on distribution, the vessels of the urinary bladder and colon should be closely examined.

The discovery of association between microhabitat and genotype is relevant to associated pathological lesions, effects on the host, and detection. Neurospirorchiidiasis is a concern in Florida turtles based on anecdotal evidence from clinical cases encountered in rehabilitation (C. Manire - personal communication) and the potential role of these parasites as a confounding health problem during a mass mortality event (Jacobson et al., 2006). Two genotypes were associated with most neurological infections in Florida *C. caretta*, one of which was found in voucher specimens of *N. schistosomatoides*. The other genotype was genetically similar but was characterized by 16 nucleotide differences in the ITS (94.7% homology) and 30 position differences (92.0% homology) in the MCOI. Based on our findings, these forms can be genetically identified when specimens for morphology are unavailable, as is most often the case,

and can be targeted by specific detection methods. The same applications are true for other forms, such as those genotypes associated with endocrine organ tropisms. Severe injury to the thyroid gland and thymus was observed in 12.0% (9/75) stranded *C. caretta* and has been observed in some *C. mydas*. The findings of the present study support that genetically distinct spirorchiids have an association with these sites and will have to be specifically targeted for detection and further disease studies. Currently available antemortem diagnostic tests, which are limited to fecal examination and crude antigen ELISA, will not detect or account for the diversity observed in this study. Adaptation of genetic data to molecular diagnostics is the next most logical step in disease studies.

Genotypes and Host Species

The other notable finding in this study was apparent restriction of some *Neospirorchis* genotypes to either *C. caretta* or *C. mydas*, which mirrored the apparent host restriction in some *Hapalotrema* species and *L. learedi*. The strongest evidence in the present study was observed in the genotypes recovered from the CNS, endocrine tissues, and gastrointestinal tract. In 37 examples in which adult spirorchiids or egg masses were sequenced from the brains of *C. caretta*, none were identified as Neogen-3. Likewise, none of the four examples of neurological parasitism in *C. mydas* were associated with genotypes recovered from *C. mydas*. In conflict with these findings were the voucher specimens of *N. schistosomatoides* (Neogen-1), which were recovered from a *C. mydas*. Neogen-1 was the most common (21/37) genotype associated with neurospirorchiidiasis in Florida *C. caretta* in the present study. Examination of more spirorchiids from the CNS of *C. mydas* is necessary. With regard to the genotypes recovered from the endocrine organs and thymus, three genotypes, Neogen-4, 5, and 6, were consistently recovered from these sites in *C. caretta* in 62 examples, whereas Neogen-7 was not found in *C. caretta*. Neogen-7, and only this genotype, was recovered from endocrine organs of *C. mydas* in

17 examples. Many of the GI forms, with the exception of Neogen-13, were limited to one or the other host species. All of the genotypes associated with larger submucosal egg masses, Neogen-9-12, were only observed in *C. caretta*. The Neogen-15 also was only found in *C. caretta*. In summary, of the nineteen observed genotypes, eleven were found in *C. caretta* and seven were found in *C. mydas*, and only one example, Neogen 13, was observed in both species. Minimally, there is a significant difference in prevalence of these parasites among different host species, at least in Florida waters.

The other observations regarding host species are that three genotypes observed in *C. caretta* also were found in single examples in *L. kempi*, which are the first observations of spirorchiidiasis in this species. It was interesting that all three examples were shared with *C. caretta*, which has a similar diet and habitat.

Coevolution and Colonization: Potential Influences on Parasite Diversity

The diversity of genetically distinct *Neospirorchis* observed within individual host species in this study was surprising in hosts originating from a single geographical region. The number of similar studies of marine parasites, however, are relatively limited, thus comparable examples are likely to emerge as molecular tools are applied in the marine environment. At this time, there are critical gaps in the data needed to test specific hypotheses regarding mechanisms of diversification of spirorchiid trematodes. Foremost is the limited number of spirorchiid taxa available for co-phylogeny studies, which make it impossible to determine whether these parasites truly co-evolved with sea turtles or it parasites have diversified, at least in some instances, by host switching/capture (colonization) (Snyder, 2004). Associations that support a hypothesis of coevolution, such as congruent phylogenies and a high degree of cospeciation (Hoberg and Klassen, 2002) have yet to be demonstrated in sea turtles and spirorchiids. Nonetheless, co-speciation and colonization likely have influenced marine spirorchiid diversity

and processes, such as intrahost speciation and host-switching between related or different hosts (including intermediate hosts), must be investigated.

Intrahost speciation is a form of co-speciation that occurs when speciation evolves in a parasite, but not the host. This process suspected when multiple congeneric species are found in the same host (Hoberg, 2005). The diversity of *Neospirorchis* genotyes observed in the present study is suggestive of such a pattern; however, evidence for intrahost speciation requires phylogenetic analysis to identify parasites as sister species and to assess for evidence of co-phylogeny verses colonization (Hoberg, 2005). A more complete representation of spirorchiid taxa from other regions and from freshwater and brackish chelonians is necessary for these analyses. There are two commonly proposed mechanisms of intrahost speciation that would produce essentially identical phylogenetic patterns, and thus would be impossible to distinguish without adequate supporting biogeographical history. The first mechanism is allopatric speciation resulting from cryptic isolation events. It is proposed that parasite fauna can serve as "cryptic indicators" of isolation events for host species and may reflect unrecognized biogeographical events (Hoberg, 2005). Isolation events may be sufficient in duration to result in speciation in parasites, but too brief to produce speciation in the host due to faster parasite generation times and higher mutation rates. The second mechanism by which intrahost speciation may occur is sympatric speciation through specialization to occupy different microhabitats within or on the host. To our knowledge, the only proposed examples of niche specialization or sympatric speciation in a host are ectoparasites (Hoberg, 2005; Dabert and Mironov, 1999). Microhabitat specialization may offer selective advantages of avoiding competition (nutrient procurement and dispersal) and promoting reproduction between like

types. With regard to *Neospirorchis*, the microhabitat fidelity observed in the various genotypes may reflect intrahost niche specialization.

Colonization is a common process in the diversification of helminths of marine vertebrates (Hoberg, 2005). Morphological evidence of host switching between sea turtles and other marine taxa has been demonstrated for Pronocephalidae (Pérez-Ponce de Leon and Brooks, 2005), which is a species-rich family of digenean trematodes of sea turtles. Thus, host-switching may have influenced spirorchiid evolution as well.

Diversity of spirorchiids in sea turtles also may reflect the colonization of new intermediate hosts or co-speciation with intermediate host species. Much of the literature on host switching and co-speciation of digenean trematodes pertains to definitive hosts; however, the same evolutionary mechanisms of diversification apply to parasite-intermediate host relationships. The older paradigm that the great host specificity of digenean gastropod interaction would make host-switching improbable has been challenged by several key exceptions. Selective pressures driving adaptation to novel intermediate hosts include avoidance of intrahost competition and access to new ecological niches, which may in turn lead to speciation events. For example, *Paragonimus* species exhibit intermediate host specificity at the superfamily level (as opposed to species specificity) and it is proposed that ecological niche partitioning may be a key influence on evolution of this parasite genus (Wilke, 2000). With regard to spirorchiids, the marine environment is inhabited by an estimated 43,000 living gastropod species as compared to around 12,000 freshwater species (Nicol, 1969). The diversity of gastropod fauna within sea turtle habitats, such as seagrass beds and reefs, dwarfs that of freshwater systems. Furthermore, marine sanguinicolids (blood flukes of fish) are unique among digeneans in that they can utilize bivalves and annelids as primary intermediate hosts (reviewed

by Smith 1997a), thus vastly increasing the numbers of potential intermediate hosts. Such adaptation also may have evolved in marine spirorchiids.

Conclusions

The genetic diversity observed in marine spirorchiids has many implications on taxonomic and biogeographical relationships, parasite-host interaction, and future parasitological studies, especially those that incorporate molecular approaches. First, the genetic divergence between Atlantic and Pacific *H. postorchis* and *L. learedi* minimally may be regarded as geographical variation, but also may reflect division of these taxa into separate species. Furthermore, the two genetically distinct genotypes observed in Florida *L. learedi* suggest the presence of a second closely-related species, possibly a cryptic species. Both findings would greatly benefit from careful morphological studies, which, as of yet, have been limited to relatively small numbers of voucher specimens. The next important finding was evidence for great diversity in the genus *Neospirorchis*, as supported by the identification of many genotypes distinct from one another and from the two currently recognized species, *N. schistosomatoides* and *N. pricei*. The selective use of host organs and tissue by many forms, differences in host species, and limited morphological data support the existence of many distinct forms that likely will be eventually translated into novel species. In addition, the gastrointestinal forms were demonstrated to be a monophyletic group and genetically different from the other *Neospirorchis* to the extent that these spirorchiids ultimately may be regarded as a separate genus. In seems likely that application of this information will progress in advance of any formal taxonomic revision at the species level. The full compliment of biological and morphological data needed for ideal assessment of biological species diversity and required for formal recognition of species is unlikely to become available in the near future for many of the parasites characterized in this study.

Although we were not able to propose changes in currently assigned species or new species, with the exception of one *Hapalotrema*, many of our findings must be considered in future studies, especially investigations involving health and disease in sea turtles. The selective organ tropisms exhibited by distinct genotypes correlates with pathological lesions, thus specific methods must be used to detect and identify spirorchiids and to associate specific parasites with any effect on the host.

The library of genetic data from spirorchiids of Florida sea turtles obtained during this study may be used as a comparative reference for identification of parasites and for the correlative morphological studies needed to further define these many forms described herein. Furthermore, genetic characterization is an alternative if morphological identification is not possible and hopefully can be used to increase the number of publications, especially disease studies, in which spirorchiids are specifically identified. Also, it hoped that further efforts will be made to better define spirorchiid fauna and diversity in other geographical regions to elucidate some of the questions regarding host restriction, genetic diversity, and host-parasite interaction.

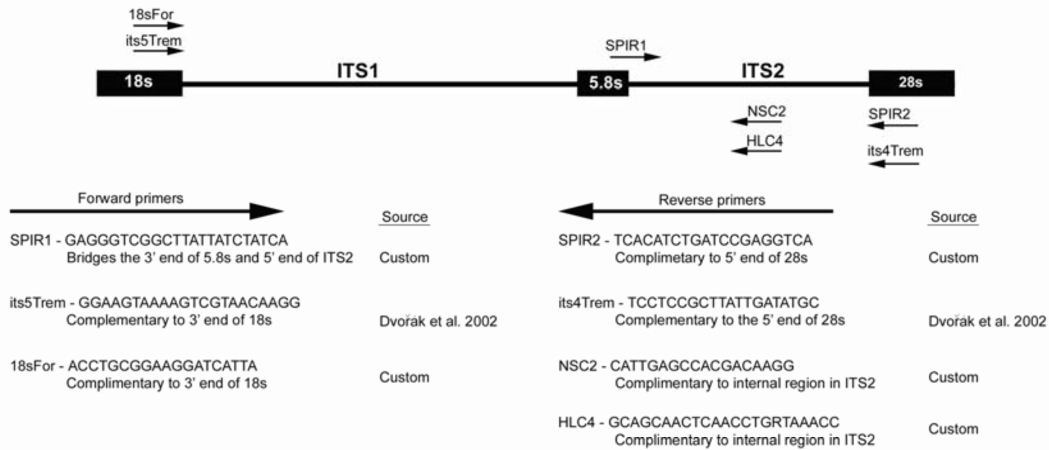


Figure 3-1. Primer sequences and binding locations for amplification of the internal transcribed spacer regions of the ribosomal gene.

```
H.postorchis.Atlantic.Caribb.       CGATGCACATTTAGTCGTGGATTGGATGAGTGCCTGCCGGCGTTGTTATC 50
H.postorchis.Hawaii                 CGACGCACATTTAGTCGTGGATTGGATGAGTGCCTGCCGGCGTTGTTATC 50
H.mistroides                        CGACGCACATTTAGTCGTGGATTGGATGAGTGCCTGCCGGCGTTGTTATC 50
Hapalotrema.novel.sp.               CGACGCACATTTAGTCGTGGATTGGATGTGTGCCTGCCGGCGTTGTTACC 50
H.pambanensis                       CGGCGCACATTTAGTCGTGGATTGGATGAGTGCCTGCCGGCGTTGTTGCC 50
                                    **  ************************ ******************  *

H.postorchis.Atlantic.Caribb.       CGTATACCTAA-TCGGATTGCTGGTCATAGGCTCCTTCCTAATTTGTCCG 99
H.postorchis.Hawaii                 CGTATACCTAA-TCGGATTGCTGGTCAAAGGCTCCTTCCTAATTTGTCCG 99
H.mistroides                        CGTATACCTAA-TCGGATTGCTGGTCAAAGGCTCCTTCCTAATTTTTCCG 99
Hapalotrema.novel.sp.               CGCATATCAAA-TCGGGTTGCTGGTCCAAGGCTCCTTCCTAATTTGTCCG 99
H.pambanensis                       CGTATAACAAAATCGGGTTGCTGGTCAAAGGCTCCTTCCTAATTTGTCCG 100
                                    ** *** * ** **** *********  ***************** ****

H.postorchis.Atlantic.Caribb.       GTGCAGCCTAATCCGGT--------TTACCAGGTTGAGTTGCTGCAATGG 141
H.postorchis.Hawaii                 GTGCAGCCTAATTCGGT--------TTACCAGGTTGAGTTGCTGCAATGG 141
H.mistroides          _             GTGCAGCCAAGTCCGGT--------TTACCAGGTTGAGTTGCTGCAATGG 141
Hapalotrema.novel.sp.               GCGCAGCCTAGTCCGGTGTTATTGTTTACCAGATTGAGTTGCTGCGGTGG 149
H.pambanensis                       GTGCAGCCTAGTCCAGT--------TTACCAGGTTGAGTTGCTGCG-TGG 141
                                    * ****** * * * **        ******* ************  ***

H.postorchis.Atlantic.Caribb.       GTATTGCTCGAGTCGTGGCTTAATGCTTTGTTTCATGCTCGAGGC----- 186
H.postorchis.Hawaii                 GTAGTGCTCGAGTCGTGGCTTAATGCTTTGTTTCATGCTCGAGGC----- 186
H.mistroides                        GTAATGCTCGAGTCGTGGCTTAATGCTTTGGTTCATGCTCGAGGC----- 186
Hapalotrema.novel.sp.               GTTATGCTCGGGTCGTGGCTTAATGTATTATTTCATGCTCGAGGCAGTTG 199
H.pambanensis                       GTTGTGCTCGAGTCATGGCTTAATACTTTGTTGCATGCTCGAGAC----- 186
                                    **  ****** *** *********   **  * ********** *

H.postorchis.Atlantic.Caribb.       ----CTATTGTGCGGCA-TATTTACACTTGATCTTGGTTTAACTGCTGTG 231
H.postorchis.Hawaii                 ----CTATTGTGCGGCA-TATTTACACTTGATCTTGGTTTAACTGCTGTG 231
H.mistroides                        ----CTATTGTGCGGCA-TATTTACACTTGATCTTGGTTTTACTGGTGCG 231
Hapalotrema.novel.sp.               AAACCTATCGTATGCTAATGTTTACACTTGATCTTGGTTCTACTGGCTAG 249
H.pambanensis                       ----CTATCGTG-GGCATAACTTACACCTTGTCTTGGTTTTGCTGACAGG 231
                                        **** **  *  *    ****** *  ********   ***    *

H.postorchis.Atlantic.Caribb.       CATGTACTGTAGGTGTGTATCACACA--ATTTATTTGACCC 270
H.postorchis.Hawaii                 CATGTACTGTAGGTGTGTAGCACACA--GTCTATTTGACCC 270
H.mistroides                        CAGGTACTGTGGGTGTGTGTCACACA--ATCTATTTGACCC 270
Hapalotrema.novel.sp.               TATGTGCTGTAGGTGTGTATCGCACATGATTCTATTGACCC 290
H.pambanensis                       CGTGTGCTGCAGATGTGCACTGCACATAATTTATTAGACCC 272
                                       ** ***  * ****     ****   *    * *****
```

Figure 3-2. Alignment of internal transcribed spacer 2 sequences of *Hapalotrema* species. Base pair differences between Hawaiian and Atlantic-Caribbean specimens of *H. postorchis* are shaded.

```
H.pambanensis.var2(1)            TCTGTATGACGTTGAGTAATAAAGATTCCCCGTTTGGTTATTATGGGCTT 50
H.pambanensis.var3(1)            TCTGTATGACGTTGAGTAATAAAGATTCCCCGTTTGGTTATTATGGGCTT 50
H.pambanensis.var1(2)            TTTGTATGACGTTGAGTAATAAAGATTCCCCGTTTGGTTATTATGGGCTT 50
H.pambanensis.var4(1)            TTTGTATGACGTTGAGTAATAAAGATTCCCCGTTTGGTTATTATGGGCTT 50
H.mistroides.var3(2)             TTTGTATGACTTTAAGAAAAAAATGATTCTTCTTTTGGTTATTATGGTTTA 50
H.mistroides.var4(1)             TTTGTATGACTTTAAGAAAAAAATGATTCTTCTTTTGGTTATTATGGTTTA 50
H.mistroides.var1(8)             TTTGTATGACTTTAAGAAAAAAATGATTCTTCTTTTGGTTATTATGGTTTA 50
H.mistroides.var5(1)             TTTGTATGACTTTAAGAAAAAAATGATTCTTCTTTTGGTTATTATGGTTTA 50
H.mistroides.var2(1)             TTTGTATGACTTTAAGAAAAAAATGATTCTTCTTTTGGTTATTATGGTTTA 50
H.postorchis.FL.var1(6)          TTTGTATGACATTGAGAAAAAATGATTCTTCCTTTGGTTATTATGGATTA 50
H.postorchis.FL.var2(1)          TTTGTATGACATTGAGAAAAAATGATTCTTCCTTTGGTTATTATGGATTA 50
H.postorchis.HW.(10)             TTTGTATGACGTTAAGGAATAATGATTCTTCATTTGGTTATTATGGGTTG 50
Hapalotrema.nov.sp.var1(3)       TTTGCATGACGTTAAGAAAAAAAGATTCATCGTTTGGTTACTATGGTCTT 50
Hapalotrema.nov.sp.var2(1)       TTTGCATGACGTTAAGAAAAAAAGATTCATCGTTTGGTTATTATGGTCTT 50
                                 * ** ***** ** ** ** ** *****  * ******** *****  *

H.pambanensis.var2(1)            GTATGTGCTATGGGTTCAATTGTTTGTTTAGGGAGAGTTGTATGGGCACA 100
H.pambanensis.var3(1)            GTATGTGCTATGGGTTCAATTGTTTGTTTAGGGAGAGTTGTATGGGCACA 100
H.pambanensis.var1(2)            GTATGTGCTATGGGTTCAATTGTTTGTTTAGGGAGAGTTGTATGGGCACA 100
H.pambanensis.var4(1)            GTATGTGCTATGGGTTCAATTGTTTGTTTAGGGAGAGTTGTATGGGCACA 100
H.mistroides.var3(2)             GTTTGTGCTATGGGTTCAATTGTCTGTTTAGGAAGTGTGGTTTGAGCTCA 100
H.mistroides.var4(1)             GTTTGTGCTATGGGTTCAATTGTCTGTTTAGGAAGTGTGGTTTGAGCTCA 100
H.mistroides.var1(8)             GTTTGTGCTATGGGTTCAATTGTCTGTTTAGGAAGTGTGGTTTGAGCTCA 100
H.mistroides.var5(1)             GTTTGTGCTATGGGTTCAATTGTCTGTTTAGGAAGTGTGGTTTGAGCTCA 100
H.mistroides.var2(1)             GTTTGTGCTATGGGTTCAATTGTCTGTTTAGGAAGTGTGGTTTGAGCTCA 100
H.postorchis.FL.var1(6)          GTGTGTGCAATGGGTTCTATAGTGTGTTTAGGGAGTGTAGTTTGAGCTCA 100
H.postorchis.FL.var2(1)_         GTGTGTGCAATGGGTTCTATAGTGTGTTTAGGGAGTGTAGTTTGAGCTCA 100
H.postorchis.HW.(10)             GTGTGTGCAATGGGTTCTATTGTGTGTTTAGGGAGTGTAGTTTGGGCTCA 100
Hapalotrema.nov.sp.var1(3)       GTTTGTGCGATGGGATCTATAGTGTGTTTAGGTAGAGTTGTTTGGGCCCA 100
Hapalotrema.nov.sp.var2(1)       GTTTGTGCGATGGGGTCTATAGTGTGTTTAGGTAGAGTTGTTTGGGCCCA 100
                                 ** ***** ***** ** ** ** ******** ** ** ** ** ** **

H.pambanensis.var2(1)            TCACATGTTTATGGTTGGGTTAGACGTAAAGACAGCAGTATTTTTTAGGT 150
H.pambanensis.var3(1)            TCACATGTTTATGGTTGGGTTAGACGTAAAGACAGCAGTATTTTTTAGGT 150
H.pambanensis.var1(2)            TCACATGTTTATGGTTGGGTTAGACGTAAAGACAGCAGTATTTTTTAGGT 150
H.pambanensis.var4(1)            TCACATGTTTATGGTTGGGTTAGACGTAAAGACAGCAGTATTTTTTAGGT 150
H.mistroides.var3(2)             TCATATGTTCATGGTTGGATTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.mistroides.var4(1)             TCATATGTTCATGGTTGGATTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.mistroides.var1(8)             TCATATGTTCATGGTTGGATTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.mistroides.var5(1)             TCATATGTTCATGGTTGGATTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.mistroides.var2(1)             TCATATGTTCATGGTTGGATTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.postorchis.FL.var1(6)          TCATATGTTTATGGTGGGGTTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.postorchis.FL.var2(1)          TCATATGTTTATGGTGGGGTTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
H.postorchis.HW.(10)             TCACATGTTTATGGTAGGGTTAGATGTGAAGACAGCTGTTTTTTTTAGAT 150
Hapalotrema.nov.sp.var1(3)       TCATATGTTTATGGTTGGTTTAGATGTGAAGACTGCAGTGTTTTTTAGTT 150
Hapalotrema.nov.sp.var2(1)       TCATATGTTTATGGTTGGTTTAGATGTGAAGACTGCAGTGTTTTTTAGTT 150
                                 *** ***** ***** ** ***** ** ***** ** ** ******** *

H.pambanensis.var2(1)            CTGTCACGATGGTTATTGGTATTCCGACAGGGATCAAGGTTTTTTCGTGA 200
H.pambanensis.var3(1)            CTGTCACGATGGTTATTGGTATTCCGACAGGGATCAAGGTTTTTTCATGA 200
H.pambanensis.var1(2)            CTGTCACGATGGTTATTGGTATTCCGACAGGGATCAAGGTTTTTTCGTGA 200
H.pambanensis.var4(1)            CTGTCACGATGGTTATTGGTATTCCGACAGGGATCAAGGTTTTTTCGTGA 200
H.mistroides.var3(2)             CTGTGACTATGGTTATAGGTATTCCTACAGGGATAAAGGTGTTTTCTTGG 200
H.mistroides.var4(1)             CTGTGACTATGGTTATAGGTATTCCTACAGGGATAAAGGTGTTTTCTTGG 200
H.mistroides.var1(8)             CTGTGACTATGGTTATAGGTATTCCTACAGGGATAAAGGTGTTTTCTTGG 200
H.mistroides.var5(1)             CTGTGACTATGGTTATAGGTATTCCTACAGGGATAAAGGTGTTTTCTTGG 200
H.mistroides.var2(1)             CTGTGACTATGGTTATAGGTATTCCTACAGGGATAAAGGTGTTTTCTTGG 200
H.postorchis.FL.var1(6)          CTGTAACTATGGTTATTGGTATACCTACAGGGATAAAGGTTTTTTCTTGA 200
H.postorchis.FL.var2(1)          CTGTAACTATGGTTATTGGTATACCTACAGGGATAAAGGTTTTTTCTTGA 200
H.postorchis.HW.(10)             CTGTAACGATGGTTATTGGAATACCTACTGGGATAAAGGTTTTTTCTTGG 200
Hapalotrema.nov.sp.var1(3)       CTGTCACAATGGTTATAGGGATACCAACTGGGATTAAGGTTTTTTCTTGA 200
Hapalotrema.nov.sp.var2(1)       CTGTCACAATGGTTATAGGGATACCAACTGGGATTAAGGTTTTTTCTTGA 200
                                 **** ** ******** ** ** ** ** ***** ***** ***** **
```

Figure 3-3. Alignment of partial nucleotide sequences of the mitochondrial cytochrome oxidase I gene of *Hapalotrema* species. Shaded positions are differences between specimens identified as the same species by morphology.

```
H.pambanensis.var2(1)          GTGTATATGCTTGGTGTCAGACGAGTGCGAATGAGAGATCCTATAGTTTG 250
H.pambanensis.var3(1)          GTGTATATGCTTGGTGTCAGACGAGTGCGAATGAGAGATCCTATAGTTTG 250
H.pambanensis.var1(2)          GTGTATATGCTTGGTGTCAGACGAGTGCGAATGAGAGATCCTATAGTTTG 250
H.pambanensis.var4(1)          GTGTATATGCTTGGTGTCAGACGAGTGCGAATGAGAGATCCTATAGTTTG 250
H.mistroides.var3(2)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGCTAGTGATCCTATAGTGTG 250
H.mistroides.var4(1)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGCTAGTGATCCTATAGTGTG 250
H.mistroides.var1(8)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGCTAGTGATCCTATAGTGTG 250
H.mistroides.var5(1)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGCTAGTGATCCTATAGTGTG 250
H.mistroides.var2(1)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGCTAGTGATCCTATAGTGTG 250
H.postorchis.FL.var1(6)        GTGTATATGTTGGGTGTTAGACGAGTTCGGGCAAGTGATCCTATAGTATG 250
H.postorchis.FL.var2(1)        GTGTATATGTTGGGTGTTAGACGAGTTCGGGCAAGTGATCCTATAGTATG 250
H.postorchis.HW.(10)           GTGTATATGTTAGGTGTTAGACGAGTTCGGGTGAGTGATCCTATAGTGTG 250
Hapalotrema.nov.sp.var1(3)     GTTTATATGTTAGGGGTTAGACGAGTACGGATGAGGGATCCAATTGTTTG 250
Hapalotrema.nov.sp.var2(1)     GTTTATATGTTAGGGGTTAGACGAGTACGGATGAGGGATCCAATTGTTTG 250
                               ** ****** * ** ** ******** **    ** ***** ** ** **

H.pambanensis.var2(1)          ATGGGTAGTAGGGTTTATTTTTCTATTTACTGTGGGGGGGGTTACTGGTA 300
H.pambanensis.var3(1)          ATGGGTAGTAGGGTTTATTTTTCTATTTACTGTGGGGGGGGTTACTGGTA 300
H.pambanensis.var1(2)          ATGGGTAGTAGGGTTTATTTTTCTATTTACTGTGGGGGGGGTTACTGGTA 300
H.pambanensis.var4(1)          ATGGGTGGTAGGGTTTATTTTTCTATTTACTGTGGGGGGGGTTACTGGTA 300
H.mistroides.var3(2)           ATGGGTTGTTGGGTTTATTTTTTTGTTTACGGTGGGGGGGGTTACTGGGA 300
H.mistroides.var4(1)           ATGGGTTGTTGGGTTTATTTTTTTGTTTACAGTGGGGGGGGTTACTGGGA 300
H.mistroides.var1(8)           ATGGGTTGTTGGGTTTATTTTTTTGTTTACTGTGGGGGGGGTTACTGGGA 300
H.mistroides.var5(1)           ATGGGTTGTTGGGTTTATTTTTTTATTTACTGTGGGGGGGGTTACTGGGA 300
H.mistroides.var2(1)           ATGGGTTGTTGGGTTTATTTTTTTGTTTACTGTAGGGGGGGTTACTGGGA 300
H.postorchis.FL.var1(6)        ATGAGTGATAGGTTTTATATTTTTGTTTACGGTAGGGGGGGTGACTGGAA 300
H.postorchis.FL.var2(1)        ATGAGTGATAGGTTTTATATTTTTGTTTACAGTAGGGGGGGTGACTGGAA 300
H.postorchis.HW.(10)           ATGAGTAGTTGGATTTATATTTTTGTTTACTGTGGGGGGGGTTACTGGAA 300
Hapalotrema.nov.sp.var1(3)     ATGGGTAGTAGGGTTTATATTTTTGTTCACTGTGGGAGGTGTAACGGGGA 300
Hapalotrema.nov.sp.var2(1)     ATGGGTAGTAGGGTTTATATTTTTGTTCACTGTGGGGGGTGTAACGGGGA 300
                               *** **  * ** ***** *** * ** ** ** ** ** ** ** ** *

H.pambanensis.var2(1)          TAGTTTTATCAGCATCTCTCTTGGATATAGTTTTTCACGATACTTGGTTT 350
H.pambanensis.var3(1)          TAGTTTTATCAGCATCTCTCTTGGATATAGTTTTTCACGATACTTGGTTT 350
H.pambanensis.var1(2)          TAGTTTTATCAGCATCTCTCTTGGATATAGTTTTTCACGATACTTGGTTT 350
H.pambanensis.var4(1)          TAGTTTTATCAGCATCTCTCTTGGATATAGTTTTTCACGATACTTGGTTT 350
H.mistroides.var3(2)           TAGTTTTATCTGCGTCGTTGTTGGATATTTTATTTCATGATACTTGGTTT 350
H.mistroides.var4(1)           TAGTTTTATCTGCGTCGTTGTTGGATATTTTATTTCATGATACTTGGTTT 350
H.mistroides.var1(8)           TAGTTTTATCTGCGTCGTTGTTGGATATTTTATTTCATGATACTTGGTTT 350
H.mistroides.var5(1)           TAGTTTTATCTGCGTCGTTGTTGGATATTTTATTTCATGATACTTGGTTT 350
H.mistroides.var2(1)           TAGTTTTATCTGCGTCGTTGTTGGATATTTTATTTCATGATACTTGGTTT 350
H.postorchis.FL.var1(6)        TAGTTTTATCTGCATCTTTATTGGATATAATTTTTCATGATACTTGATTT 350
H.postorchis.FL.var2(1)        TAGTTTTATCTGCATCTTTATTGGATATAATTTTTCATGATACTTGATTT 350
H.postorchis.HW.(10)           TAGTTTTATCTGCGTCTTTATTAGATATAATTTTTCATGATACTTGGTTT 350
Hapalotrema.nov.sp.var1(3)     TTGTTTTATCAGCTTCTTTGTTGGATGTTATTTTTCATGATACTTGATTC 350
Hapalotrema.nov.sp.var2(1)     TTGTTTTATCAGCTTCTTTGTTGGATGTTATTTTTCATGATACTTGATTC 350
                               * ******** ** **  * ** *** *  * ***** ******** **

H.pambanensis.var2(1)          GTGATAGCTCATTTTCA 367
H.pambanensis.var3(1)          GTGATAGCTCATTTTCA 367
H.pambanensis.var1(2)          GTGATAGCTCATTTTCA 367
H.pambanensis.var4(1)          GTGATAGCTCATTTTCA 367
H.mistroides.var3(2)           GTTATTGCTCATTTTCA 367
H.mistroides.var4(1)           GTTATTGCTCATTTTCA 367
H.mistroides.var1(8)           GTTATTGCTCATTTTCA 367
H.mistroides.var5(1)           GTTATTGCTCATTTTCA 367
H.mistroides.var2(1)           GTTATTGCTCATTTTCA 367
H.postorchis.FL.var1(6)        GTTATTGCTCATTTTCA 367
H.postorchis.FL.var2(1)        GTTATTGCTCATTTTCA 367
H.postorchis.HW.(10)           GTTATAGCTCATTTTCA 367
Hapalotrema.nov.sp.var1(3)     GTAATAGCTCATTTTCA 367
Hapalotrema.nov.sp.var2(1)     GTAATAGCTCATTTTCA 367
                               ** ** ***********
```

Figure 3-3. Continued.

```
H.pambanensis.MCOI              CMTLSNNDSPFGYYGLVCAMGSIVCLGSVVWAHHMFMVGLDVKTAVFFSS 50
H.mistroides.MCOI               CMTLSNNDSSFGYYGLVCAMGSIVCLGSVVWAHHMFMVGLDVKTAVFFSS 50
H.postorchis.FL.MCOI            CMTLSNNDSSFGYYGLVCAMGSMVCLGSVVWAHHMFMVGLDVKTAVFFSS 50
H.postorchis.HW.MCOI            CMTLSNNDSSFGYYGLVCAMGSIVCLGSVVWAHHMFMVGLDVKTAVFFSS 50
Hapalotrema.nov.sp.MCOI         CMTLSNNDSSFGYYGLVCAMGSMVCLGSVVWAHHMFMVGLDVKTAVFFSS 50
                                *********.************:***************************

H.pambanensis.MCOI              VTMVIGIPTGIKVFSWVYMLGVSRVRMSDPMVWWVVGFIFLFTVGGVTGM 100
H.mistroides.MCOI               VTMVMGIPTGMKVFSWVYMLGVSRVRASDPMVWWVVGFIFLFTVGGVTGM 100
H.postorchis.FL.MCOI            VTMVIGMPTGMKVFSWVYMLGVSRVRASDPMVWWVMGFMFLFTVGGVTGM 100
H.postorchis.HW.MCOI            VTMVIGMPTGMKVFSWVYMLGVSRVRVSDPMVWWVVGFMFLFTVGGVTGM 100
Hapalotrema.nov.sp.MCOI         VTMVMGMPTGIKVFSWVYMLGVSRVRMSDPIVWWVVGFMFLFTVGGVTGI 100
                                ****:*:***:*************** ***:****:**:**********:

H.pambanensis.MCOI              VLSASLLDMVFHDTWFVMAHF 121
H.mistroides.MCOI               VLSASLLDILFHDTWFVIAHF 121
H.postorchis.FL.MCOI            VLSASLLDMIFHDTWFVIAHF 121
H.postorchis.HW.MCOI            VLSASLLDMIFHDTWFVMAHF 121
Hapalotrema.nov.sp.MCOI         VLSASLLDVIFHDTWFVMAHF 121
                                ********::*******:***
```

Figure 3-4. Alignment of partial predicted amino acid sequence of mitochondrial cytochrome oxidase I gene of *Hapalotrema* species. Shaded positions are differences between specimens identified as the same species by morphology.

```
L.learedi.variant A          GTCGGCTTATTATCTATCACGGCGCACATTTAGTCGTGGATTGGATGAGT 50
L.learedi.variant B          GTCGGCTTATTATCTATCACGGCGCACATTTAGTCGTGGATTGGATGAGT 50
                             **************************************************

L.learedi.variant A          GCCTGCCGGCGTTGTTACCCGTATAACAAAATCGGGTTGCTGGTCAAAGG 100
L.learedi.variant B          GCCTGCCGGCGTTGTTACCCGTATAACAAAATCGGGTTGCTGGTCAAAGG 100
                             **************************************************

L.learedi.variant A          CTCCTTCCTAATTTGTCCGGCGCAGCCTAGTCCGGTTTATCAGGTTGAGT 150
L.learedi.variant B          CTCCTTCCTAATTTGTCCGGCGCAGCCTTGTCCGGTTTATCAGGTTGAGT 150
                             **************************** *********************

L.learedi.variant A          TGCTGCGGTGGGTTGTGCTCGAGTCGTGGCTTAATGCTTTGTTTCATGCT 200
L.learedi.variant B          TGCTGCGGTGGGTTGTGCTCGAGTCGTGGCTTAATGCTTTGTTTCATGCT 200
                             **************************************************

L.learedi.variant A          CGGGACCTATCGTGTGCATCATTTATGCCTCAGCTTGGTTTCACTGGCAG 250
L.learedi.variant B          CGGGACCTATCGTGTGCATCATTTATGCCTCAGCTTGGTTTTACTGGCAG 250
                             ***************************************** ********

L.learedi.variant A          GTATGTGCTGCAGGTGTGCATCACACATTTCCCAATTTGACCCTGACCT 299
L.learedi.variant B          GTATGTGCTGCAGGTGTGCATCACACATTTCCCAATTTGACCCTGACCT 299
                             *************************************************
```

Figure 3-5. Alignment of internal transcribed spacer 2 (ITS2) sequences of *Learedius learedi*.

```
L.learedi.MCOI.var1       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGACAC 50
L.learedi.MCOI.var2       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGACAC 50
L.learedi.MCOI.var3       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTTAGACAC 50
L.learedi.MCOI.var4       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGACAC 50
L.learedi.MCOI.var5       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGGCAC 50
L.learedi.MCOI.var6       ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGACAC 50
L.learedi.MCOI.var7       ATCCTGAGGTTTATGTTTTAATTCTACCTGGGTTTGGTGTGGTAAGACAT 50
L.learedi.MCOI.var8       ATCCTGAGGTTTATGTTTTAATTCTACCTGGGTTTGGTGTGGTAAGACAT 50
L.learedi.MCOI.var9       ATCCTGAGGTTTATGTTTTAATTCTACCTGGGTTTGGTGTGGTAAGACAT 50
L.learedi.MCOI.var10      ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTGAGACAT 50
L.learedi.MCOI.var11      ATCCTGAGGTTTATGTTTTAATTCTACCTGGATTTGGTGTGGTAAGACAT 50
L.learedi.MCOI.var12      ATCCTGAGGTTTATGTTTTAATTCTACCAGGGTTTGGAATAGTAAGTCAT 50
L.learedi.MCOI.var13      ATCCTGAGGTTTATGTTTTAATTCTGCCTGGGTTTGGGGTAGTAAGTCAT 50
                          ************************* ** ** *****  * ** ** ** 

L.learedi.MCOI.var1       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var2       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var3       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var4       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var5       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var6       ATTTGTATGACTTTGAGAAATAATGATTCTACGTTTGGTTATTATGGTCT 100
L.learedi.MCOI.var7       ATTTGTATGACTTTAAGGAATAATGACTCTACGTTTGGTTATTATGGGCT 100
L.learedi.MCOI.var8       ATTTGTATGACTTTAAGGAAAAATGACTCTACGTTTGGTTATTATGGGCT 100
L.learedi.MCOI.var9       ATTTGTATGACTTTAAGGAATAATGATTCTACGTTTGGTTATTATGGGCT 100
L.learedi.MCOI.var10      ATTTGTATGACTTTAAGTAATAATGATTCTACGTTTGGTTATTATGGACT 100
L.learedi.MCOI.var11      ATTTGTATGACTTTAAGTAATAATGATTCTACGTTTGGTTATTATGGACT 100
L.learedi.MCOI.var12      ATTTGTATGACTTTAAGTAATAATGATTCTACGTTTGGTTATTATGGACT 100
L.learedi.MCOI.var13      ATTTGTATGACCTTAAGTAATAATGATTCTACGTTTGGTTATTATGGACT 100
                          *********** ** ** ** ***** ******************** **

L.learedi.MCOI.var1       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var2       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var3       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var4       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var5       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var6       TGTTTGTGCTATGGGTTCAATAGTGTGTTTGGGTAGTGTGGTATGAGCTC 150
L.learedi.MCOI.var7       TGTTTGTGCTATGGGTTCAATAGTGTGCTTAGGAAGTGTAGTGTGGGCTC 150
L.learedi.MCOI.var8       TGTTTGTGCTATGGGTTCAATAGTGTGCTTAGGAAGTGTAGTGTGGGCTC 150
L.learedi.MCOI.var9       TGTTTGTGCTATGGGTTCAATAGTGTGCTTAGGAAGTGTAGTGTGGGCTC 150
L.learedi.MCOI.var10      TGTTTGTGCTATGGGTTCAATAGTCTGTTTAGGAAGTGTAGTGTGGGCTC 150
L.learedi.MCOI.var11      TGTTTGTGCTATGGGTTCAATAGTCTGTCTAGGAAGTGTAGTGTGGGCTC 150
L.learedi.MCOI.var12      TGTTTGTGCTATGGGTTCAATAGTGTGTTTAGGAAGCGTAGTATGGGCTC 150
L.learedi.MCOI.var13      TGTTTGTGCTATGGGTTCAATAGTGTGTTTAGGGAGTGTAGTGTGGGCTC 150
                          ************************ **  * ** ** ** ** ** ****

L.learedi.MCOI.var1       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var2       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var3       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var4       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var5       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var6       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCTGTTTTTTTTAGT 200
L.learedi.MCOI.var7       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
L.learedi.MCOI.var8       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
L.learedi.MCOI.var9       ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
L.learedi.MCOI.var10      ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
L.learedi.MCOI.var11      ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
L.learedi.MCOI.var12      ATCATATGTTTATGGTGGGTTTGGATGTTAAGACTGCAATATTTTTTAGT 200
L.learedi.MCOI.var13      ATCATATGTTTATGGTTGGTTTAGATGTTAAGACTGCAGTTTTTTTTAGT 200
                          **************** ***** **************  * *********

L.learedi.MCOI.var1       TCTGTAACTATGGTTATTGGGATCCCCACAGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var2       TCTGTAACTATGGTTATTGGGATCCCCACGGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var3       TCTGTAACTATGGTTATTGGGATCCCCACGGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var4       TCTGTAACTATGGTTATTGGGATCCCCACAGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var5       TCTGTAACTATGGTTATTGGGATCCCCACAGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var6       TCTGTAACTATGGTTATTGGGATTCCCACAGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var7       TCTGTAACTATGGTTATTGGAATTCCTACTGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var8       TCCGTAACTATGGTTATTGGAATTCCTACTGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var9       TCTGTCACTATGGTTATTGGAATTCCTACTGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var10      TCTGTAACTATGGTTATTGGAATTCCTACTGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var11      TCTGTAACTATGGTTATTGGAATTCCTACTGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var12      TCTGTAACTATGGTTATTGGAATTCCTACAGGAATAAAGGTTTTTTCTTG 250
L.learedi.MCOI.var13      TCTGTAACTATGGTTATTGGAATTCCTACGGGAATAAAGGTTTTTTCTTG 250
                          ** ** ************* ** ** ** *********************
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Figure 3-6. Mitochondrial cytochrome oxidase I gene partial nucleotide sequence for *Learedius learedi*.

```
L.learedi.MCOI.var1           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var2           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var3           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var4           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var5           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var6           AATATATATGCTTGGAGTTAGTCGAGTGCGGGCTAAAGATCCCATAGTTT 300
L.learedi.MCOI.var7           AATATATATGCTTGGTGTTAGTCGAGTACGGGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var8           AATATATATGCTTGGTGTTAGTCGAGTACGGGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var9           AATATATATGCTTGGTGTTAGTCGAGTACGGGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var10          AATATACATGCTTGGTGTTAGTCGAGTGCGAGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var11          AATATACATGCTTGGTGTTAGTCGAGTGCGAGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var12          AATATATATGCTTGGAGTTAGTCGAGTACGGGCTAATGATCCTATAGTTT 300
L.learedi.MCOI.var13          GATATATATGCTTGGAGTTAGTCGAGTACGGGCTAATGATCCTATAGTTT 300
                               ***** ******** *********** ** ***** ***** *******

L.learedi.MCOI.var1           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var2           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTGACTGGT 350
L.learedi.MCOI.var3           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var4           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var5           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var6           GATGAATAGTTGGTTTTATTTTTTTGTTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var7           GATGAATAGTTGGATTTATTTTTTTATTTACTGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var8           GATGAATAGTTGGATTTATTTTTTTATTTACTGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var9           GATGAATAGTTGGATTTATTTTTTTATTTACCGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var10          GATGAATAGTTGGATTTATTTTTTTATTTACTGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var11          GATGAATAGTTGGATTTATTTTTTTATTTACTGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var12          GATGAATAGTTGGGTTTATTTTTTTATTTACGGTGGGGGGGGTAACTGGT 350
L.learedi.MCOI.var13          GATGAATAGTTGGGTTTATTTTTTTATTTACTGTGGGGGGGGTAACAGGT 350
                              ************* *********** ***** *********** ** ***

L.learedi.MCOI.var1           ATTGTTTTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var2           ATTGTTTTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var3           ATTGTTTTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var4           ATTGTTCTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var5           ATTGTTCTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var6           ATTGTTCTATCTGCATCTTTGTTGGATATATTATTCCATGATACTTGGTT 400
L.learedi.MCOI.var7           ATTGTTTTATCTGCATCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var8           ATTGTTCTATCTGCATCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var9           ATTGTTCTATCTGCATCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var10          ATTGTTTTATCTGCATCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var11          ATTGTTTTATCTGCATCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var12          ATTGTTTTATCTGCGTCTCTTTTGGATATATTATTTCATGATACTTGATT 400
L.learedi.MCOI.var13          ATTGTTTTATCTGCATCTCTTTTAGATATATTATTTCATGATACTTGATT 400
                              ****** ******* *** * ** *********** *********** **

L.learedi.MCOI.var1           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var2           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var3           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var4           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var5           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var6           TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var7           TGTAATAGCTCATTTTCA 418
L.learedi.MCOI.var8           TGTAATAGCTCATTTTCA 418
L.learedi.MCOI.var9           TGTAATAGCTCATTTTCA 418
L.learedi.MCOI.var10          TGTAATAGCTCATTTTCA 418
L.learedi.MCOI.var11          TGTAATAGCTCATTTTCA 418
L.learedi.MCOI.var12          TGTGATAGCTCATTTTCA 418
L.learedi.MCOI.var13          CGTGATAGCTCATTTTCA 418
                               ** **************
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Figure 3-6. Continued.

```
L.learedi.MCOI.variant A    PEVYVLILPGFGVVSHICMTLSNNDSTFGYYGLVCAMGSMVCLGSVVWAH 50
L.learedi.MCOI.variant B    PEVYVLILPGFGMVSHICMTLSNNDSTFGYYGLVCAMGSMVCLGSVVWAH 50
                            ************:*************************************

L.learedi.MCOI.variant A    HMFMVGLDVKTAVFFSSVTMVIGIPTGMKVFSWMYMLGVSRVRANDPMVW 100
L.learedi.MCOI.variant B    HMFMVGLDVKTAMFFSSVTMVIGIPTGMKVFSWMYMLGVSRVRANDPMVW 100
                            ************:*************************************

L.learedi.MCOI.variant A    WMVGFIFLFTVGGVTGIVLSASLLDMLFHDTWFVMAHF 138
L.learedi.MCOI.variant B    WMVGFIFLFTVGGVTGIVLSASLLDMLFHDTWFVMAHF 138
                            **************************************
```

Figure 3-7. Alignment of partial amino acid sequences of the mitochondrial cytochrome oxidase I gene of *Learedius learedi*.

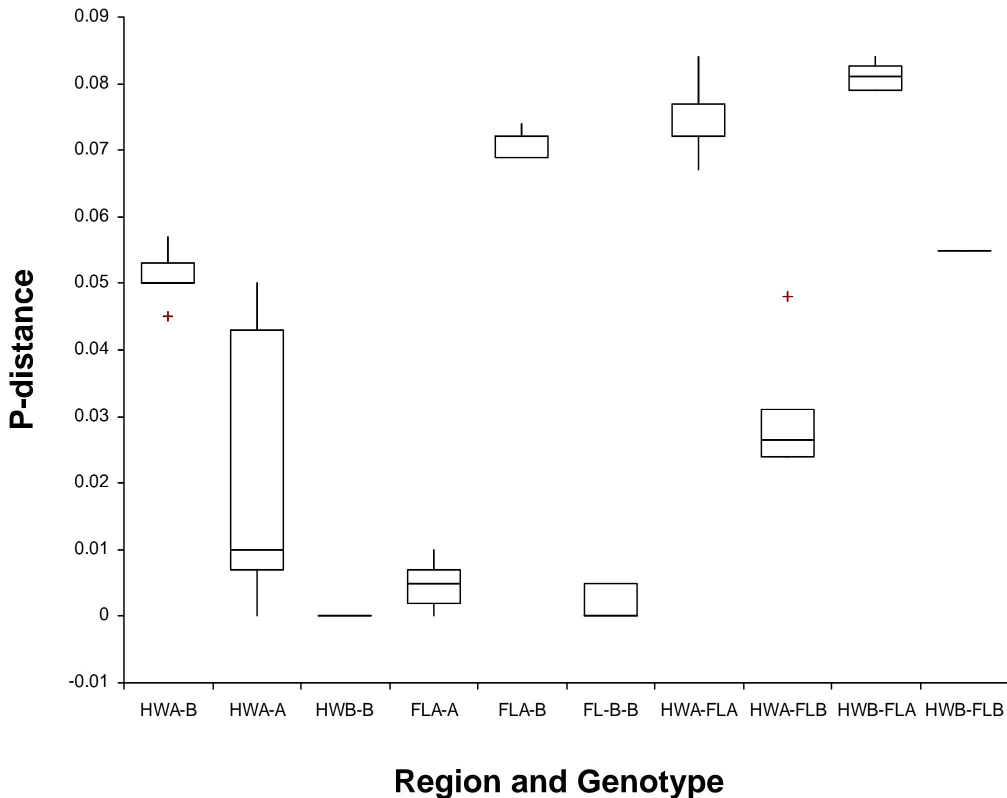

Figure 3-8. Box and whiskers plot of pairwise distances in the mitochondrial cytochrome oxidase I gene within and between genotype variants of *L.learedi* from Florida and Hawaii. Outliers are denoted by a plus sign. Note the significantly lower pairswise distances between Hawaii variant A and Florida variant B as compared to genotypes from the same locality.

```
05-51.C.bipora.ITS2        CGATGTACATTAAGTCGTGGATTGGATGTGTGCCTGCCGGCAGTTATACT 50
05-58.C.bipora.ITS2        CGATGTACATTAAGTCGTGGATTGGATGTGTGCCTGCCGGCAGTTATACT 50
06-12.C.bipora.1.ITS2      CGATGTACATTAAGTCGTGGATTGGATGTGTGCCTGCCGGCAGTTATACT 50
06-12.C.bipora.3.ITS2      CGATGTACATTAAGTCGTGGATTGGATGTGTGCCTGCCGGCAGTTATACT 50
06-12.C.bipora.2.ITS2      CGATGTACATTAAGTCGTGGATTGGATGTGTGCCTGCCGGCAGTTATACT 50
                           **************************************************

05-51.C.bipora.ITS2        CGTATATCAACGCGAGTTGTCGGTCTAAGGCTCCTTCCTAATTTGTCCGG 100
05-58.C.bipora.ITS2        CGTATATCAACGCGAGTTGTCGGTCTAAGGCTCCTTCCTAATTTGTCCGG 100
06-12.C.bipora.1.ITS2      CGTATATCAACGCGAGTTGTCGGTCTAAGGCTCCTTCCTAATTTGTCCGG 100
06-12.C.bipora.3.ITS2      CGTATATCAACGCGAGTTGTCGGTCTAAGGCTCCTTCCTAATTTGTCCGG 100
06-12.C.bipora.2.ITS2      CGTATATCAACGCGAGTTGTCGGTCTAAGGCTCCTTCCTAATTTGTCCGG 100
                           **************************************************

05-51.C.bipora.ITS2        CTCAGCCTAGTCCGCAATGAAGACCAGACTGAATTGTTACAGTGGGTTGT 150
05-58.C.bipora.ITS2        CTCAGCCTAGTCCGCAATGAAGACCAGACTGAATTGTTACAGTGGGTTGT 150
06-12.C.bipora.1.ITS2      CTCAGCCTAGTCCGCAATGAAGACCAGACTGAATTGTTACAGTGGGTTGT 150
06-12.C.bipora.3.ITS2      CTCAGCCTAGTCCGCAATGAAGACCAGACTGAATTGTTACAGTGGGTTGT 150
06-12.C.bipora.2.ITS2      CTCAGCCTAGTCCGCAATGAAGACCAGACTGAATTGTTACAGTGGGTTGT 150
                           **************************************************

05-51.C.bipora.ITS2        GCTTGAGTCATGGGTTAATGTTGATATACATGCTCGCACAGTAAGCCCCT 200
05-58.C.bipora.ITS2        GCTTGAGTCATGGGTTAATGTTGATATACATGCTCGCACAGTAAGCCCCT 200
06-12.C.bipora.1.ITS2      GCTTGAGTCATGGGTTAATGTTGATATACATGCTCGCACAGTAAGCCCCT 200
06-12.C.bipora.3.ITS2      GCTTGAGTCATGGGTTAATGTTGATATACATGCTCGCACAGTAAGCCCCT 200
06-12.C.bipora.2.ITS2      GCTTGAGTCATGGGTTAATGTTGATATACATGCTCGCACAGTAAGCCCCT 200
                           **************************************************

05-51.C.bipora.ITS2        ACTGTTTTCATCTTGGTTCTGAAACGGTCTATGGCTTGTACCGAGAGTGC 250
05-58.C.bipora.ITS2        ACTGTTTTCATCTTGGTTCTGAAACGGTCTATGGCTTGTACCGAGAGTGC 250
06-12.C.bipora.1.ITS2      ACTGTTTTCATCTTGGTTCTGAAACGGTCTATGGCTTGTACCGAGAGTGC 250
06-12.C.bipora.3.ITS2      ACTGTTTTCATCTTGGTTCTGAAACGGTCTATGGCTTGTACCGAGAGTGC 250
06-12.C.bipora.2.ITS2      ACTGTTTTCATCTTGGTTCTGAAACGGTCTATGGCTTGTACCGAGAGTGC 250
                           **************************************************

05-51.C.bipora.ITS2        ATAAGGCACAGTGTCTATTTTATCC 275
05-58.C.bipora.ITS2        ATAAGGCACAGTGTCTATTTTATCC 275
06-12.C.bipora.1.ITS2      ATAAGGCACAGTGTCTATTTTATCC 275
06-12.C.bipora.3.ITS2      ATAAGGCACAGTGTCTATTTTATCC 275
06-12.C.bipora.2.ITS2      ATAAGGCACAGTGTCTATTTTATCC 275
                           *************************
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Figure 3-9. Alignment of internal transcribed spacer 2 (ITS2) sequences of *Carettacola bipora*.

```
06-12.C.bipora.1.MCOI      TTTGGGCATCCTGAGGTTTATGTTTTAATTTTACCTGGGTTTGGTATGGT 50
06-12.C.bipora.3.MCOI      TTTGGGCATCCTGAGGTTTATGTTTTAATTTTACCTGGGTTTGGTATGGT 50
06-12.C.bipora.2.MCOI      TTTGGGCATCCTGAGGTTTATGTTTTAATTTTACCTGGGTTTGGTATGGT 50
                           **************************************************

06-12.C.bipora.1.MCOI      TAGACATATTTGTATGAGGTTGAGTAAAAATGATTCTTCTTTTGGGTATT 100
06-12.C.bipora.3.MCOI      TAGACATATTTGTATGAGGTTGAGTAAAAATGATTCTTCTTTTGGGTATT 100
06-12.C.bipora.2.MCOI      TAGACATATTTGTATGAGGTTGAGTAAAAATGATTCTTCTTTTGGGTATT 100
                           **************************************************

06-12.C.bipora.1.MCOI      ATGGGTTGGTGTGTGCTATGGGGGCTATAGTATGTTTGGGGAGTGTGGTT 150
06-12.C.bipora.3.MCOI      ATGGGTTGGTGTGTGCTATGGGGGCTATAGTATGTTTGGGGAGTGTGGTT 150
06-12.C.bipora.2.MCOI      ATGGGTTGGTGTGTGCTATGGGGGCTATAGTATGTTTGGGGAGTGTGGTT 150
                           **************************************************

06-12.C.bipora.1.MCOI      TGAGCGCATCATATGTTTATGATTGGTTTAGATATTAAGACTGCTGTGTT 200
06-12.C.bipora.3.MCOI      TGAGCGCATCATATGTTTATGATTGGTTTAGATATTAAGACTGCTGTGTT 200
06-12.C.bipora.2.MCOI      TGAGCGCATCATATGTTTATGATTGGTTTAGATATTAAGACTGCTGTGTT 200
                           **************************************************

06-12.C.bipora.1.MCOI      TTTTAGTTCAGTTACTATGGTAATAGGGATTCCTACTGGGATAAAGATAT 250
06-12.C.bipora.3.MCOI      TTTTAGTTCAGTTACTATGGTAATAGGGATTCCTACTGGGATAAAGATAT 250
06-12.C.bipora.2.MCOI      TTTTAGTTCAGTTACTATGGTAATAGGGATTCCTACTGGGATAAAGATAT 250
                           **************************************************

06-12.C.bipora.1.MCOI      TTTCTTGATTGTATATGCTTGGTGTTAGTAATATTCGTGTTAATGATCCA 300
06-12.C.bipora.3.MCOI      TTTCTTGATTGTATATGCTTGGTGTTAGTAATATTCGTGTTAATGATCCA 300
06-12.C.bipora.2.MCOI      TTTCTTGATTGTATATGCTTGGTGTTAGTAATATTCGTGTTAATGATCCA 300
                           **************************************************

06-12.C.bipora.1.MCOI      ATTGTTTGGTGGATTTTAGGGTTTATTTTTTTATTTACTATTGGTGGGGT 350
06-12.C.bipora.3.MCOI      ATTGTTTGGTGGATTTTAGGGTTTATTTTTTTATTTACTATTGGTGGGGT 350
06-12.C.bipora.2.MCOI      ATTGTTTGGTGGATTTTAGGGTTTATTTTTTTATTTACTATTGGTGGGGT 350
                           **************************************************

06-12.C.bipora.1.MCOI      TACTGGGATTGTTTTATCTGCTTCAGTTTTGGATAGTTTGTTTCATGATA 400
06-12.C.bipora.3.MCOI      TACTGGGATTGTTTTATCTGCTTCAGTTTTGGATAGTTTGTTTCATGATA 400
06-12.C.bipora.2.MCOI      TACTGGGATTGTTTTATCTGCTTCAGTTTTGGATAGTTTGTTTCATGATA 400
                           **************************************************

06-12.C.bipora.1.MCOI      CTTGGTTTATAATTGCTCATTTTCATT 427
06-12.C.bipora.3.MCOI      CTTGGTTTATAATTGCTCATTTTCATT 427
06-12.C.bipora.2.MCOI      CTTGGTTTATAATTGCTCATTTTCATT 427
                           ***************************
```

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HPEVYVLILPGFGMVSHICMSLSNNDSSFGYYGLVCAMGAMVCLGSVVWAHHMFMIGLDIKTAVFFSSVTMVMGIPTGMKMFSWLYMLGVSNIRVND
PIVWWILGFIFLFTIGGVTGIVLSASVLDSLFHDTWFMIAHFH
```

Figure 3-10. Mitochondrial cytochrome oxidase I gene partial nucleotide sequence and predicted amino acid sequence for *Carettacola bipora*.

```
Neogen1         CGACGCACAATTA-GTCGTGGCTTGGATGCTGTGCCAACCGGCGTGTTACTCTCACATAC 59
Neogen2         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTGCTCTCACATAC 59
Neogen3         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCTGCATGTTACTCTCACATAC 59
Neogen4         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCGTGTTACTCTCACATAC 59
Neogen5         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTACTCTCACATAC 59
Neogen6         CGACGCACAACTA-GTCGTGGCTTGGATGTTGTGCCAACTGGCATGTTACTCTCACATAC 59
Neogen7         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTACTCTCACATAC 59
Neogen8         CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCTTGTTGCTCTCACATAC 59
Neogen9         CGGCGCACAATTT-GTCGTGGCTTGGATGCTGTGCCAACCGGCCTGTTTATCTCACGT-- 57
Neogen10        CGACGCACAATTT-GTCGTGGCTTGGATGCTGTGCCAACCGGCCTGCTTTGCTCACTT-- 57
Neogen11        CGGCGCACAATTT-GTCGTGGCTTGGATGCTGTGCCAACCGGCCTGTTTATCTCACGT-- 57
Neogen12        CGACGCACAATTT-GTCGTGGCTTGGATGTTGTGCCAACCAGCCTGAT-TTCTCA----- 53
Neogen13        CGACGCACAATTT-GTCGTGGCTTGGATGCTGTGCCAACCGGCCTGTTTCCCCCATGTAT 59
Neogen14        CGACGCACAATTTTGTCGTGGCTTGGATGCTGTGCCAACCGGCCTGTTTCCCCCATGTAT 60
Neogen15        CGACGCACAAATT-GTCGTGGCTTGGATGCTGTGCCAACCGGCATGGTTAGCTCAATTGT 59
Neogen16        CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTACTCTCACATAC 59
Neogen17        CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTACTCTCACATAC 59
Neogen18        CGACGCACAATTA-GTCGTGGCTTGGATGTTGTGCCAACCGGCATGTTACTCTCACATAC 59
                ** ******* *  *************** *********  ** ** *   * **

Neogen1         TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen2         TGT---------GGTTGAATGTATGAGTTTGAGAGTCGCTGGTTTAATGGCTCCATCCCA 110
Neogen3         TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen4         TGTTGTTTTTGTTGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 119
Neogen5         TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen6         TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen7         TGT------TGTTGTTGGATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 113
Neogen8         TGT---------GATTGGATGTGTGTAATTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen9         -------------GTCCATGGTATACGGTTGTGGGATGCTGGTTTAATGGCTCCATCCTA 104
Neogen10        -------------GTCCGTCGTATACGGTTGTGAGATGCCGGTTTAATGGCTCCATCCTA 104
Neogen11        -------------ATCCATGGTATACGGTTGTGGGATGCTGGTTTAATGGCTCCATCCTA 104
Neogen12        -------------------TGTGTAAGGTTGTGGGAAGCTGGTTTAATGGCTCCATCCTA 94
Neogen13        CCG--------TAGTGCAATGTGCGGTTGTGGG---TGCTGGTTTAATGGCTCCATCCTA 108
Neogen14        CCG--------TGGTATAATGTGCGGTTGTGGG---TGCTGGTTTAATGGCTCCATCCCA 109
Neogen15        CCG--------TGGTATAATATACGG--TTGTGGGCTGCCGGTTTAATGGCTCCATCCTA 109
Neogen16        TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCCATCCCA 110
Neogen17        TGT---------GGTTGAATGTATGAGTTTGAGAGTTGCTGGTTTAATGGCTCAAGCCCA 110
Neogen18        TGT---------GATT-AATGTATGAGTTTGAGAGTTGCTGGTTTCATGGCTCCATCCCA 109
                                     *       ** *    ** ****  ******* * ** *

Neogen1         ATGTGTCCGGTTGCAACGTTGTTCGGGTCGAGTGGCTCGTCTGGGTTGTGACGTTGGGTT 170
Neogen2         ATGTGTCCAGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGGCGATGGGTT 170
Neogen3         ATGTGTCCAGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGGCGATGGGTT 170
Neogen4         ATGTGTCCAGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGACGGTAGGTT 179
Neogen5         ATGTGTCCAGTTGCAACATTGTTCGGGTCGGGCGGCTCGTCTGGGTTGTGACGATGGGTT 170
Neogen6         ATGTGTCCGGTTGCAACGTTGTTCGGGTCGGATGGCTCGTCTGGGTTGTGACGTTGGGTT 170
Neogen7         ATGTGCCCAGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGACGGTGGGTT 173
Neogen8         ATGTGTCCAGTTGCAACGTTGTTCGGGTCGGATGGCTTGACTGGGTTGTGGCGATGGGTT 170
Neogen9         ATGTGTCCAGCTGCAACATTGTTCGGGTCTGATGGCTCGTTTGGGTTGTAGCGATGGGTT 164
Neogen10        ATGTGTCCAGCTGCAACGTTGCTTGGGTCTGATGACTTGTTTGGGTTGTGGCGTTGGGTT 164
Neogen11        ATGTGTCCAGCTGCAACATTGTTCGGGTCTGATGGCTCGTTTGGGTTGTAGCGATGGGTT 164
Neogen12        ATGTGTCCAGCTGCAACATTGCTTGGGTCTTGTGGCTCGTTTGGGTTGTGGCGGTGGGTT 154
Neogen13        ATGTGTCCAGCTGCAACATTACTCGGGTCTGATGGCCTGTTTGGGTTGTGGCGATGGGTT 168
Neogen14        ATGTGTCCAGCTGCAACATTGTTCGGGTCTGATGGCCTGTTGGGGTTGTGGCGATGGGTT 169
Neogen15        ATGTGTCCAGCTGCAAGATTGCCCAGATCTAATGGTTTGATTGAATTCTGGCGGCGGGCT 169
Neogen16        ATGTGTCCGGTTACAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGACGTTGGGTT 170
Neogen17        ATGTGTCCGGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGACGTTGGGTT 170
Neogen18        ATGTGTCCAGTTGCAACGTTGTTCGGGTCGGGTGGCTCGTCTGGGTTGTGGCGGTGGGTT 169
                ***** ** * * ***  **     * **    *    *   *  ** *  **   ** *
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Figure 3-11. Alignment of internal transcribed spacer 2 sequences of *Neospirorchis* specimens.

```
Neogen1         GTGCACCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTATAATCTGTCGTTTGC 230
Neogen2         GTGCCCCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTATAAGCTGTCGTTTGT 230
Neogen3         ATGCACCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTGTAGTCTGTCGTTTGC 230
Neogen4         TTACGCCTTGTCGTGGCTCAATGATTTGGATCACGTTTGGTGTTATAATCTGTCGTTTGC 239
Neogen5         GTGCACCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTATGATCTGTCGTTTGT 230
Neogen6         GTGCACCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTGCAATCTGTCGTTTGT 230
Neogen7         GTGCACCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTATAATCTGTCGTTTGT 233
Neogen8         GTGCGCCTTGTCGTGGCTCAATGTTTTGGATCACGCTTGGTGTTATGATCTGTTGTTCAT 230
Neogen9         TTGC-CCTTGTCGTGGCTCAATGTTGTTGATCACGCTTGGTGTCATGATCTGTCGTTCAT 223
Neogen10        GTGC-CCTTGTCGTGGCTCAATGTTTT-GATCACGCTTGGTGTCATGGTCCATCGTTCAT 222
Neogen11        TTGC-CCTTGTCGTGGCTCAATGTTGTTGATCACGCTTGGTGTCTTGATCTGTCGTTCAT 223
Neogen12        GTGC-CCTTGTCGTGGCTCAATGTTGTTGATCACGCTTGGTGTCATGGTCTGTCGTTCAT 213
Neogen13        ATGC-CCTTGTCGTGGCTCAATGTTTTTGATCACGCTTGGTGTCATGGTCTGTCGTCTCA 227
Neogen14        ATGC-CCTTGTCGTGGCTCAATGTTTTTGATCACGCTTGGTGTCATGATCTGTCGTCTCA 228
Neogen15        ATAC-------------------------------------------------------- 173
Neogen16        ATGCGCCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTTTAATCTGTCGTTTAT 230
Neogen17        GTGCGCCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTATAATCTGTCGTTTGT 230
Neogen18        ATGCTCCTTGTCGTGGCTCAATGATTTGGATCACGCTTGGTGTTGTAATTTGTCGTTTGT 229
                 * * ****************** * * ******* *******  *      * **

Neogen1         GACCTTTTCTTGCTATTCTGATT-TGGCAATTGTTTGTGGCTGATGAGCTT-TAAAAGCT 288
Neogen2         GGCCCTTTCTTGCTATTCTGATT-TGGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen3         AACTCTTTCTTGCTATTCTGATT-TAGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen4         GCCCCTTTCTTGCTATTCGGACTGTGGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 298
Neogen5         GACTCTTTCTTGCTATTCTGATT-TGGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen6         GACCCTTTCTTGCTATTCTGATT-TGGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen7         GATCCTTTCTTGCTATTCGGACTGTGGCAATTGTTTGTGGCTGAAGAGCTT-TGAAAGCT 292
Neogen8         GGCTCTCTCTTGCTGTTTTGATT-TGGCAATTGTCTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen9         GGCTCTTTCTTGCTATTATGATC-TGAAAATCCCTTGCAGTTGATGAGCATTTAAAAGCT 282
Neogen10        AGCTCTTTCTTGTTATTTTGATC-CGGGAATCGCTTGTGGCTGATGAGCATTTGGAAGCT 281
Neogen11        GGCTCTTTCTTGCTATTATGATC-TGGGAATCCCTTGCAGTTGATGAGCATTTAAAAGCT 282
Neogen12        ATCTCTTTCTTGCTATTATGATC-TGGCAGTGGCTTGTGGCTGATGAGCTT-TGAAAAGC 271
Neogen13        TTCTCTTTCTTGCTATTATGATC-TGGGAATTGCTTGTGGCTGATGAGCTCCTGAGAGCT 286
Neogen14        TTCTCTTTCTTGCCATTATGATC-TGGGAATTGTTTGTGGTTGATGGGCTCCTGAGAGCT 287
Neogen15        ------------------------------------------------------------
Neogen16        GGCCCTTTCTTGCTATTCTGCCT-TGGCAATTGTTTGTGGCTGATGAGCTT-TGAAAGCT 288
Neogen17        GACCTTTTCTTGCTATTCTGATT-TGGCAACTGTTTGTAGCTGATGAGCTT-TAAAAGCT 288
Neogen18        GACCCTTTCTTGCTATTCTGATT-TGGCAATTGTTTGTGGCTGATGAGCTT-TTGAAGCT 287
                     *******   **  *                *    *** * **   *

Neogen1         GTT--TCTGTTGACCC 302
Neogen2         AGT--TCTGTTGACCC 302
Neogen3         ATT--TCTGTTGACCC 302
Neogen4         ATT--TCTGTTGACCC 312
Neogen5         ATT--TCTGTTGACCC 302
Neogen6         ATT--TCTGTTGACCC 302
Neogen7         ATT--TCTGTTGACCC 306
Neogen8         AAT--ATTGTTGACCC 302
Neogen9         TTA-ACCTGTTGACCC 297
Neogen10        TTTATCTTGTTGACCC 297
Neogen11        TTA-ACCTGTTGACCC 297
Neogen12        TTTCATTTGTTGACCC 287
Neogen13        TTA--TCTGTTTATCC 300
Neogen14        TTA--TCTGTTGACCC 301
Neogen15        ----------------
Neogen16        ATT--TCTGTTGACCC 302
Neogen17        ATT--TCTGTTGACCC 302
Neogen18        ATT--TATGTTGACCC 301
                       ****   **
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Figure 3-11. Continued.

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NEOGEN8.I.MCOI.NTD(6)                                         TGAGTCATATATGTA 15
NEOGEN8.II.MCOI.NTD(1)                                        TGAGTCATATATGTA 15
NEOGEN8.III.MCOI.NTD(1)                                       TGAGTCATATATGTA 15
NEOGEN1.MCOI.NTD(18)        GGTTTATGTTTTAATTCTTCCAGGGTTTGGAATGGTTAGTCATATTTGTA 50
NEOGEN17.MCOI.NTD(1)                                                ATATTTGTA 9
NEOGEN7.MCOI.NTD(36)        GGTTTATGTGTTAATTCTTCCTGGGTTTGGGATGGTTAGTCATATTTGTA 50
NEOGEN4.MCOI.NTD(42)        GGTTTATGTATTAATTCTTCCAGGGTTTGGAATGGTTAGTCATATTTGTA 50
NEOGEN2.MCOI.NTD(13)        GGTTTATGTTTTAATTCTTCCAGGTTTTGGAATGGTTAGTCATATTTGTA 50
NEOGEN3.I.MCOI.NTD(3)       GGTTTATGTTTTAATTCTTCCCGGGTTTGGTATGGTTAGTCATATTTGTA 50
NEOGEN3.II.MCOI.NTD(1)      GGTTTATGTTTTAATTCTTCCTGGGTTTGGGATGGTTAGTCATATTTGTA 50
NEOGEN6.MCOI.NTD(1)         GGTTTATGTTTTGATTCTTCCAGGGTTTGGTATGGTTAGTCATATTTGTA 50
NEOGEN19.MCOI.NTD(2)        GGTTTATGTTTTGATTCTTCCTGGATTTGGTATGGTTAGTTACATTTGTA 50
NEOGEN18.MCOI.NTD(4)        GGTTTATGTTTTAATTCTTCCAGGGTTTGGGATGGTTAGTCATATTTGTA 50
NEOGEN5.I.MCOI.NTD(3)       GGTTTATGTTTTGATTCTTCCAGGTTTTGGAATGGTTAGTCATATTTGTA 50
NEOGEN5.II.MCOI.NTD(8)      GGTTTATGTTTTGATTCTTCCGGGTTTTGGGATGGTTAGTCATATTTGTA 50
NEOGEN9.I.MCOI.NTD(9)       GGTTTATGTTTTGATAATTCCTGGTTTTGGTATGGTTAGACATATATGTA 50
NEOGEN9.II.MCOI.NTD(2)      GGTTTATGTTTTGATAATTCCTGGTTTTGGTATGGTTAGACATATATGTA 50
NEOGEN11.MCOI.NTD(8)        GGTTTATGTTTTGATAATTCCTGGTTTTGGTATGGTTAGACATATATGTA 50
NEOGEN13.I.MCOI.NTD(2)      GGTTTATGTGTTGATTCTACCTGGTTTTGGTATGGTTAGTCATATATGTA 50
NEOGEN13.II.MCOI.NTD(1)     GGTTTATGTGTTGATTTTACCTGGTTTTGGTATGGTTAGTCATATATGTA 50
NEOGEN14.I.MCOI.NTD(2)      GGTTTATGTTTTAATTCTTCCTGGTTTTGGTATGGTTAGTCATATTTGTA 50
NEOGEN14.II.MCOI.NTD(3)     GGTTTATGTTTTAATTCTTCCTGGTTTTGGTATGATTAGTCATATTTGTA 50
NEOGEN10.MCOI.NTD(11)       GGTTTATGTTTTAATTCTTCCAGGGTTTGGTATGGTTAGACATATATGTA 50
NEOGEN15.I.MCOI.NTD(1)      GGTTTATGTTTTAATTCTTCCTGGGTTTGGGATGGTTAGACATATATGTA 50
NEOGEN15.II.MCOI.NTD(3)     GGTTTATGTTTTAATTCTTCCTGGGTTTGGGATGGTTAGACATATATGTA 50
NEOGEN12.MCOI.NTD(4)        GGTTTATGTTTTGATTCTTCCTGGTTTTGGTATGGTTAGTCACATATGTA 50
                            ********* ** **  * ** ** ***** ***** **  * ** ****

NEOGEN8.I.MCOI.NTD(6)       TGACTTTGAGTAATAAAGAGTCTATGTTTGGTTATTTTGGTTTAGTGTGT 65
NEOGEN8.II.MCOI.NTD(1)      TGACTTTGAGTAACAAAGAGTCTATGTTTGGTTATTTTGGTTTAGTGTGT 65
NEOGEN8.III.MCOI.NTD(1)     TGACTTTGAGTAATAAAGAGTCTATGTTTGGTTATTTTGGTTTAGTGTGT 65
NEOGEN1.MCOI.NTD(18)        TGACTTTAAGAAAAAGAGAGTCTTTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN17.MCOI.NTD(1)        TGACTTTGAGAAAAAAAGAGTCTTTGTTTGGTTATTTTGGTTTGGTTTGT 59
NEOGEN7.MCOI.NTD(36)        TGACTTTAAGGAATAATGAGTCATTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN4.MCOI.NTD(42)        TGACTTTGAGGAATAATGAATCGTTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN2.MCOI.NTD(13)        TGACTTTGAGGAATAATGAGTCGTTGTTTGGTTACTTTGGTTTGGTTTGT 100
NEOGEN3.I.MCOI.NTD(3)       TGACTCTAAGAAATAATGAGTCATTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN3.II.MCOI.NTD(1)      TGACTTTAAGAAATAATGAGTCGTTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN6.MCOI.NTD(1)         TGACTTTAAGAAAAAAAGAGTCTTTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN19.MCOI.NTD(2)        TGACTTTAAGGAATAGTGAGTCGTTATTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN18.MCOI.NTD(4)        TGACTTTAAGGAATAATGAGTCGTTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN5.I.MCOI.NTD(3)       TGACTTTAAGAAATAATGAGTCGTTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN5.II.MCOI.NTD(8)      TGACTTTAAGAAATAATGAGTCGTTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN9.I.MCOI.NTD(9)       TGGTTTTAAGGAAAAGTGAGTCTGTATTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN9.II.MCOI.NTD(2)      TGGTTTTAAGGAAAAGTGAGTCTGTATTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN11.MCOI.NTD(8)        TGGTTTTAAGGAAAAGTGAGTCTGTGTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN13.I.MCOI.NTD(2)      TGGTTTTAAGGAAAAATGAGTCGGTGTTTGGTTATTTTGGTTTAGTGTGT 100
NEOGEN13.II.MCOI.NTD(1)     TGGTTTTAAGGAAAAATGAGTCGGTGTTTGGTTATTTTGGTTTAGTGTGT 100
NEOGEN14.I.MCOI.NTD(2)      TGGTTTTAAGAAAAAATGAGTCGGTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN14.II.MCOI.NTD(3)     TGGTTTTAAGAAAAAATGAGTCGGTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN10.MCOI.NTD(11)       TGGTTTTAAGTAATAATGAGTCTGTGTTTGGTTATTTTGGTTTGGTTTGT 100
NEOGEN15.I.MCOI.NTD(1)      TGACTTTAAGAAAAAGTGAGTCTGTTTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN15.II.MCOI.NTD(3)     TGACTTTAAGAAAAAGTGAGTCTGTTTTTGGTTATTTTGGTTTAGTTTGT 100
NEOGEN12.MCOI.NTD(4)        TGGTTTTGAGAAAAAGGGAGTGTGTGTTTGGTTATTTTGGATTGGTTTGT 100
                            **  * * ** ** *  ** *   * ******** ***** ** ** ***
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Figure 3-12. Alignment of partial nucleotide sequences of the mitochondrial cytochrome oxidase I gene for *Neospirorchis* specimens. Shaded positions are differences between like genotypes based on predicted amino acid (mitochondrial cytochrome oxidase I) sequence and ribosomal ITS2 sequence.

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NEOGEN8.I.MCOI.NTD(6)         GCTATGGGTGCTATTGTTTGTTTAGGTAGTATAGTTTGGGCTCATCATAT 115
NEOGEN8.II.MCOI.NTD(1)        GCTATGGGTGCTATTGTTTGTTTAGGTAGTATAGTTTGGGCTCATCATAT 115
NEOGEN8.III.MCOI.NTD(1)       GCTATGGGTGCTATTGTTTGTTTAGGTAGTATAGTTTGGGCTCATCATAT 115
NEOGEN1.MCOI.NTD(18)          GCTATGGGTGCTATAGTTTGTCTAGGTAGAATAGTTTGGGCTCATCATAT 150
NEOGEN17.MCOI.NTD(1)          GCTATGGGTGCTATTGTATGTTTAGGTAGGATAGTTTGAGCTCATCATAT 109
NEOGEN7.MCOI.NTD(36)          GCTATGGGTGCTATTGTTTGTTTGGGTAGTATAGTTTGGGCTCATCATAT 150
NEOGEN4.MCOI.NTD(42)          GCTATGGGTGCTATTGTTTGTTTGGGTAGTATAGTTTGGGCTCATCATAT 150
NEOGEN2.MCOI.NTD(13)          GCTATGGGTGCTATTGTTTGTTTAGGTAGTATAGTTTGGGCTCATCACAT 150
NEOGEN3.I.MCOI.NTD(3)         GCTATGGGTGCTATAGTTTGTTTAGGTAGAATAGTTTGAGCTCATCATAT 150
NEOGEN3.II.MCOI.NTD(1)        GCTATGGGTGCTATAGTCTGTTTGGGTAGAATAGTTTGGGCTCATCATAT 150
NEOGEN6.MCOI.NTD(1)           GCTATGGGTGCTATAGTTTGTTTGGGTAGAATAGTCTGGGCTCATCATAT 150
NEOGEN19.MCOI.NTD(2)          GCTATGGGTGCTATTGTTTGTTTGGGTAGAATAGTTTGAGCTCATCATAT 150
NEOGEN18.MCOI.NTD(4)          GCTATGGGTGCTATTGTTTGTTTGGGTAGGATAGTTTGAGCTCATCATAT 150
NEOGEN5.I.MCOI.NTD(3)         GCTATGGGTGCTATAGTTTGTTTGGGTAGGATAGTTTGAGCTCATCATAT 150
NEOGEN5.II.MCOI.NTD(8)        GCTATGGGTGCTATAGTTTGTTTGGGTAGAATAGTTTGAGCTCATCATAT 150
NEOGEN9.I.MCOI.NTD(9)         GCTATGGGAGCAATAGTATGTTTAGGAAGGATAGTTTGAGCTCATCATAT 150
NEOGEN9.II.MCOI.NTD(2)        GCTATGGGAGCAATAGTATGTTTAGGAAGGATAGTTTGAGCTCATCATAT 150
NEOGEN11.MCOI.NTD(8)          GCTATGGGAGCGATAGTGTGTTTAGGTAGGATAGTTTGAGCTCATCATAT 150
NEOGEN13.I.MCOI.NTD(2)        GCTATGGGTGCTATTGTGTGTTTGGGAAGTATAGTTTGAGCTCATCATAT 150
NEOGEN13.II.MCOI.NTD(1)       GCTATGGGTGCTATTGTGTGTTTGGGAAGTATAGTTTGAGCTCATCATAT 150
NEOGEN14.I.MCOI.NTD(2)        GCTATGGGGGCTATAGTATGTTTGGGTAGAATTGTTTGAGCCCATCACAT 150
NEOGEN14.II.MCOI.NTD(3)       GCTATGGGGGCTATAGTATGTTTGGGTAGGATTGTTTGAGCTCATCACAT 150
NEOGEN10.MCOI.NTD(11)         GCAATGGGAGCTATAGTGTGTTTAGGTAGAATAGTGTGGGCTCATCATAT 150
NEOGEN15.I.MCOI.NTD(1)        GCTATGGGGGCTATAGTTTGTTTAGGTAGAATAGTTTGGGCGCATCATAT 150
NEOGEN15.II.MCOI.NTD(3)       GCTATGGGGGCTATAGTTTGTTTAGGTAGAATAGTTTGGGCGCATCATAT 150
NEOGEN12.MCOI.NTD(4)          GCTATGGGTTCAATTGTATGTTTGGGTAGTGTGGTTTGGGCGCATCATAT 150
                              ** *****  * ** ** *** * ** **  * ** ** ** ***** **

NEOGEN8.I.MCOI.NTD(6)         GTTTGTTGTTGGTATGGATATAAAGACTGCCGTTTTTTTTAGGTCTGTTA 165
NEOGEN8.II.MCOI.NTD(1)        GTTTGTTGTTGGTATGGATATAAAGACTGCCGTTTTTTTTAGGTCTGTTA 165
NEOGEN8.III.MCOI.NTD(1)       GTTTGTTGTTGGTATGGATATAAAGACTGCCGTTTTTTTTAGGTCTGTTA 165
NEOGEN1.MCOI.NTD(18)          GTTTGTTGTTGGTATGGATATAAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN17.MCOI.NTD(1)          GTTTGTTGTTGGTATGGATGTTAAGACTGCTGTGTTTTTTAGGTCAGTTA 159
NEOGEN7.MCOI.NTD(36)          GTTTGTTGTGGGTATGGATATTAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN4.MCOI.NTD(42)          GTTTGTTGTTGGTATGGATATTAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN2.MCOI.NTD(13)          GTTTGTTGTGGGTATGGATATAAAGACTGCTGTGTTTTTTAGGTCTGTTA 200
NEOGEN3.I.MCOI.NTD(3)         GTTTGTTGTGGGTATGGATATTAAGACTGCTGTGTTTTTTAGGTCTGTTA 200
NEOGEN3.II.MCOI.NTD(1)        GTTTGTTGTGGGTATGGATATTAAGACTGCTGTATTTTTTAGATCTGTTA 200
NEOGEN6.MCOI.NTD(1)           GTTTGTTGTTGGTATGGATATTAAGACTGCTGTGTTTTTTAGATCAGTTA 200
NEOGEN19.MCOI.NTD(2)          GTTTGTTGTGGGTATGGATATTAAGACTGCTGTTTTTTTTAGGTCGGTTA 200
NEOGEN18.MCOI.NTD(4)          GTTTGTTGTGGGTATGGATATTAAGACTGCTGTGTTTTTTAGGTCTGTTA 200
NEOGEN5.I.MCOI.NTD(3)         GTTTGTTGTGGGTATGGATATAAAGACTGCTGTGTTTTTTAGGTCAGTTA 200
NEOGEN5.II.MCOI.NTD(8)        GTTTGTTGTGGGTATGGATATAAAGACTGCTGTGTTTTTTAGGTCAGTTA 200
NEOGEN9.I.MCOI.NTD(9)         GTTTGTGATTGGTATGGATATAAAGACTGCTGTTTTTTTTAGGTCTGTTA 200
NEOGEN9.II.MCOI.NTD(2)        GTTTGTGATTGGTATGGATATAAAGACTGCTGTTTTTTTTAGGTCTGTTA 200
NEOGEN11.MCOI.NTD(8)          GTTTGTGGTTGGGATGGATATAAAGACTGCTGTTTTTTTTAGATCTGTTA 200
NEOGEN13.I.MCOI.NTD(2)        GTTTGTTGTTGGTATGGATATAAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN13.II.MCOI.NTD(1)       GTTTGTTGTTGGTATGGATATAAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN14.I.MCOI.NTD(2)        GTTTGTTGTTGGAATGGATATAAAGACTGCTGTGTTTTTTAGGTCTGTTA 200
NEOGEN14.II.MCOI.NTD(3)       GTTTGTTGTTGGAATGGATATAAAGACTGCTGTGTTTTTTAGATCTGTTA 200
NEOGEN10.MCOI.NTD(11)         GTTTGTGATTGGTATGGATATAAAGACTGCTGTGTTTTTTAGCTCTGTTA 200
NEOGEN15.I.MCOI.NTD(1)        GTTTGTGGTTGGGATGGATGTTAAGACTGCTGTTTTTTTTAGTTCCGTTA 200
NEOGEN15.II.MCOI.NTD(3)       GTTTGTGGTTGGGATGGATGTTAAGACTGCTGTTTTTTTTAGTTCCGTTA 200
NEOGEN12.MCOI.NTD(4)          GTTTGTAATTGGGATGGATTTGAAGACTGCTGTATTTTTTAGTTCGGTTA 200
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Figure 3-12. Continued.

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NEOGEN8.I.MCOI.NTD(6)         CTATGATTATTGGTATTCCTACAGGTATTAAGGTATTCTCTTGATTATAT 215
NEOGEN8.II.MCOI.NTD(1)        CTATGATTATTGGTATTCCTACAGGTATTAAGGTATTCTCTTGATTATAT 215
NEOGEN8.III.MCOI.NTD(1)       CTATGATTATTGGTATTCCTACAGGTATTAAGGTATTCTCTTGATTATAT 215
NEOGEN1.MCOI.NTD(18)          CTATGATAATTGGTATTCCTACAGGTATAAAGGTGTTTTCTTGGTTATAT 250
NEOGEN17.MCOI.NTD(1)          CTATGATAATTGGTATTCCTACTGGTATAAAGGTGTTTTCTTGGTTGTAT 209
NEOGEN7.MCOI.NTD(36)          CGATGATAATTGGAATTCCAACTGGTATAAAGGTGTTTTCCTGATTGTAT 250
NEOGEN4.MCOI.NTD(42)          CAATGATAATTGGGATTCCGACTGGTATAAAGGTGTTTTCATGGTTATAT 250
NEOGEN2.MCOI.NTD(13)          CTATGATTATTGGTATTCCGACTGGTATAAAGGTGTTTTCTTGATTGTAT 250
NEOGEN3.I.MCOI.NTD(3)         CTATGATAATTGGTATTCCAACAGGAATAAAGGTGTTTTCTTGGTTATAT 250
NEOGEN3.II.MCOI.NTD(1)        CTATGATAATTGGTATTCCAACAGGTATAAAGGTGTTTTCTTGGTTATAT 250
NEOGEN6.MCOI.NTD(1)           CTATGATAATTGGTATTCCTACAGGTATAAAGGTTTTTTCTTGGTTGTAT 250
NEOGEN19.MCOI.NTD(2)          CTATGATAATTGGTATTCCTACGGGGATAAAGGTGTTTTCTTGGTTGTAT 250
NEOGEN18.MCOI.NTD(4)          CTATGATAATTGGTATTCCGACTGGTATAAAGGTGTTTTCTTGATTATAT 250
NEOGEN5.I.MCOI.NTD(3)         CTATGATTATTGGTATTCCGACAGGTATAAAGGTATTTTCTTGGTTATAT 250
NEOGEN5.II.MCOI.NTD(8)        CTATGATTATTGGTATTCCGACAGGTATAAAGGTGTTTTCTTGGTTATAT 250
NEOGEN9.I.MCOI.NTD(9)         CTATGATTATTGGTGTTCCTACAGGTATAAAGGTTTTTTCTTGATTGTAT 250
NEOGEN9.II.MCOI.NTD(2)        CTATGATTATTGGTGTTCCTACAGGTATAAAGGTTTTTTCTTGATTGTAT 250
NEOGEN11.MCOI.NTD(8)          CGATGATTATTGGTGTTCCTACAGGTATAAAGGTTTTTTCTTGATTGTAT 250
NEOGEN13.I.MCOI.NTD(2)        CGATGATTATTGGTATTCCTACAGGTATAAAGGTTTTTTCTTGGTTGTAT 250
NEOGEN13.II.MCOI.NTD(1)       CGATGATTATTGGTATTCCTACAGGTATAAAGGTTTTTTCTTGGTTGTAT 250
NEOGEN14.I.MCOI.NTD(2)        CTATGATTATAGGTGTTCCTACAGGTATAAAGGTTTTTTCTTGACTATAT 250
NEOGEN14.II.MCOI.NTD(3)       CTATGATTATAGGCGTTCCTACAGGTATAAAGGTTTTTTCTTGATTATAT 250
NEOGEN10.MCOI.NTD(11)         CTATGATTATTGGTATACCGACCGGGATTAAGGTTTTTTCTTGATTATAT 250
NEOGEN15.I.MCOI.NTD(1)        CTATGATAATTGGTATACCTACGGGGATAAAGGTTTTTTCTTGATTGTTT 250
NEOGEN15.II.MCOI.NTD(3)       CTATGATAATTGGTATACCTACGGGGATAAAGGTTTTTTCTTGATTGTTT 250
NEOGEN12.MCOI.NTD(4)          CTATGATTATAGGTGTTCCGACCGGAATTAAGGTTTTTTCGTGGTTGTAT 250
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NEOGEN8.I.MCOI.NTD(6)         ATGCTTGGTAGAAGATATGTTCGGTTGGTTGATCCTGTGGTATGATGAAT 265
NEOGEN8.II.MCOI.NTD(1)        ATGCTTGGTAGAAGATATGTTCGGTTGGTTGATCCTGTGGTATGATGAAT 265
NEOGEN8.III.MCOI.NTD(1)       ATGCTTGGTAGAAGATATGTTCGGTTGGTTGATCCTGTGGTATGATGAAT 265
NEOGEN1.MCOI.NTD(18)          ATGCTTGGTAGGAGATATGTTCGGTTGGTTGATCCTGTAGTTTGGTGGAT 300
NEOGEN17.MCOI.NTD(1)          ATGCTTGGTAGTAGATATGTTCGGTTGGTTGATCCTGTAGTTTGGTGAAT 259
NEOGEN7.MCOI.NTD(36)          ATGCTTGGTAGCAGATACGTTCGGTTAGTTGATCCGGTGGTTTGGTGAAT 300
NEOGEN4.MCOI.NTD(42)          ATGCTTGGTAGGAGGTATGTTCGATTGGTTGATCCTGTGGTTTGATGAAT 300
NEOGEN2.MCOI.NTD(13)          ATGCTTGGTAGAAGATATGTTCGGTTGGTTGATCCAGTGGTTTGGTGAAT 300
NEOGEN3.I.MCOI.NTD(3)         ATGTTAGGTAGGAGGTATGTTCGATTAGTTGATCCAGTGGTTTGGTGAAT 300
NEOGEN3.II.MCOI.NTD(1)        ATGTTAGGTAGTAGGTATGTTCGATTAGTTGATCCAGTGGTTTGGTGAAT 300
NEOGEN6.MCOI.NTD(1)           ATGCTTGGTAGGAGGTATGTTCGTTTGGTTGATCCTGTGGTTTGGTGGGT 300
NEOGEN19.MCOI.NTD(2)          ATGTTGGGTAGTAGGTATGTTCGACTGGTTGATCCGGTGGTTTGGTGGAT 300
NEOGEN18.MCOI.NTD(4)          ATGCTTGGTAGGAGTTATGTTCGGTTGATTGATCCTGTGGTTTGATGAAT 300
NEOGEN5.I.MCOI.NTD(3)         ATGCTTGGTAGGAGGTATGTTCGATTAGTTGATCCAGTGGTTTGGTGAAT 300
NEOGEN5.II.MCOI.NTD(8)        ATGCTTGGTAGGAGGTATGTTCGATTAGTTGATCCAGTGGTTTGGTGAAT 300
NEOGEN9.I.MCOI.NTD(9)         ATGCTTGGTGGGAGTAATGTTCGTTTATTTGATCCTGTGGTTTGATGAAT 300
NEOGEN9.II.MCOI.NTD(2)        ATGCTTGGTGGGAGTAATGTTCGTTTATTTGATCCTGTGGTTTGATGAAT 300
NEOGEN11.MCOI.NTD(8)          ATGCTTGGGGGAAGAAATGTTCGTTTATTTGATCCTGTGGTTTGATGAAT 300
NEOGEN13.I.MCOI.NTD(2)        ATGCTTGGCGGTAGTGGTGTTCGATTATATGATCCTGTTGTATGGTGGGT 300
NEOGEN13.II.MCOI.NTD(1)       ATGCTTGGCGGTAGTGGTGTTCGATTATATGATCCTGTTGTATGGTGGGT 300
NEOGEN14.I.MCOI.NTD(2)        ATGCTTGGTGGGAGTGGTGTTCGTTTGTTTGATCCTGTGGTGTGGTGGGT 300
NEOGEN14.II.MCOI.NTD(3)       ATGCTTGGTGGGAGTGGTGTTCGTTTGTTTGATCCTGTGGTGTGGTGGGT 300
NEOGEN10.MCOI.NTD(11)         ATGCTTGGGAATAGTGGTGTTCGTTTATTAGATCCGGTGGTTTGGTGGGT 300
NEOGEN15.I.MCOI.NTD(1)        ATGCTTGGTAGGAGGAATATTCGTGTGTATGATCCGATAGTTTGGTGAAT 300
NEOGEN15.II.MCOI.NTD(3)       ATGCTTGGTAGGAGAAATATTCGTGTGTATGATCCGATAGTTTGGTGAAT 300
NEOGEN12.MCOI.NTD(4)          ATGTTGGGTGGAAGAGGTGTTCGTGTTTATGATCCAGTTGTTTGGTGAAT 300
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Figure 3-12. Continued.

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NEOGEN8.I.MCOI.NTD(6)          AATAGGTTTTATTTTTTTATTTACAGTGGGTGGGGTAACTGGCATAGTTT 315
NEOGEN8.II.MCOI.NTD(1)         AATAGGTTTTATTTTTTTATTTACAGTGGGTGGGGTAACTGGCATAGTTT 315
NEOGEN8.III.MCOI.NTD(1)        AATAGGTTTTATTTTTTTATTTACAGTGGGTGGGGTAACTGGGATAGTTT 315
NEOGEN1.MCOI.NTD(18)           TGTGGGTTTTATTTTTTTGTTTACTATAGGTGGTGTGACTGGGATTGTTT 350
NEOGEN17.MCOI.NTD(1)           TGTGGGTTTTATTTTTTTATTTACTATAGGCGGTGTAACTGGGATTGTTT 309
NEOGEN7.MCOI.NTD(36)           TATAGGTTTTATTTTTTTATTTACGGTTGGTGGTGTTACTGGAATTGTTT 350
NEOGEN16.MCOI.NTD(3)           TATAGGTTTTATTTTTTTATTTACGGTTGGTGGTGTTACTGGAATTGTTT 302
NEOGEN4.MCOI.NTD(42)           TATAGGTTTTATTTTTTTATTTACAGTTGGTGGTGTTACTGGGATTGTTT 350
NEOGEN2.MCOI.NTD(13)           TATAGGTTTTATTTTTTTGTTTACAATAGGTGGTGTTACTGGGATTGTTT 350
NEOGEN3.I.MCOI.NTD(3)          CATAGGTTTTATTTTTTTGTTTACTGTTGGTGGGGTTACTTGGAATTGTTT 350
NEOGEN3.II.MCOI.NTD(1)         TATAGGTTTTATTTTTTTGTTTACTGTTGGTGGGGTTACCGGGATTGTTT 350
NEOGEN6.MCOI.NTD(1)            TATAGGTTTTATTTTTTTATTTACTATAGGTGGTGTTACTGGAATTGTTT 350
NEOGEN19.MCOI.NTD(2)           TATAGGTTTTATTTTTTTGTTTAC                           324
NEOGEN18.MCOI.NTD(4)           TATAGGTTTTATTTTTTTGTTTAC                           324
NEOGEN5.I.MCOI.NTD(3)          TATAGGTTTTATTTTTTTGTTTACGATAGGTGGTGTTACTGGGATTGTTT 350
NEOGEN5.II.MCOI.NTD(8)         TATAGGTTTTATTTTTTTGTTTACAATAGGTGGTGTTACTGGGATTGTTT 350
NEOGEN9.I.MCOI.NTD(9)          TGTTGGGTTTATATTTTTGTTTACTGTAGGAGGGGTTACTGGAATAGTGT 350
NEOGEN9.II.MCOI.NTD(2)         TGTTGGGTTTATATTTTTGTTTACTGTAGGAGGGGTTACTGGAATAGTGT 350
NEOGEN11.MCOI.NTD(8)           TGTTGGGTTTATATTTTTGTTTACTGTTGGGGGAGTTACTGGGATAGTGT 350
NEOGEN13.I.MCOI.NTD(2)         GATTGGGTTTATATTTTTGTTTACAATTGGGGGTGTTACTGGTATAGTTT 350
NEOGEN13.II.MCOI.NTD(1)        GATTGGGTTTATATTTTTGTTTACAATTGGGGGTGTTACTGGTATAGTTT 350
NEOGEN14.I.MCOI.NTD(2)         TATTGGTTTTATATTTTTATTTGTTGTTGGTGGGGTTACTGGAGTTGTTT 350
NEOGEN14.II.MCOI.NTD(3)        TATTGGTTTTATATTTTTATTTGTTGTTGGTGGGGTTACTGGTATTGTTT 350
NEOGEN10.MCOI.NTD(11)          TGTTGGGTTTATATTTTTGTTTACTATTGGTGGTGTTACTGGTATAGTTT 350
NEOGEN15.I.MCOI.NTD(1)         AATTGGATTTGTGTTTTTGTTTACGATTGGTGGGATTACTGGGGTTGTAT 350
NEOGEN15.II.MCOI.NTD(3)        AATTGGATTTGTGTTTTTGTTTACGATTGGTGGGATTACTGGGGTTGTAT 350
NEOGEN12.MCOI.NTD(4)           TTTAGGCTTTGTGTTTTTATTTACAATAGGTGGGGTTACTGGAATAGTTT 350
                                 * ** *** * ***** ***    * ** **  * ** **  * ** *

NEOGEN8.I.MCOI.NTD(6)          TGTCTGCGTCGGTTGTAGACTCTTTGTTTCATGATACATGATTTGTTGTG 365
NEOGEN8.II.MCOI.NTD(1)         TGTCTGCGTCGGTTGTAGACTCTTTGTTTCATGATACATGATTTGTTGTG 365
NEOGEN8.III.MCOI.NTD(1)        TGTCTGCGTCGGTTGTAGACTCTTTGTTTCATGATACATGATTTGTTGTG 365
NEOGEN1.MCOI.NTD(18)           TATCTGCTTCGGTTATTGATTCTTTGTTTCATGATACTTGGTTTGTTGTT 400
NEOGEN17.MCOI.NTD(1)           TGTCTGCTTCGGTTATTGATTCTTTGTTTCATGATACTTGATTTGTTGTT 359
NEOGEN7.MCOI.NTD(36)           TGTCTGCTTCGGTTATTGATTCATTGTTTCATGATACTTGATTTGTTGTT 400
NEOGEN16.MCOI.NTD(3)           TGTCTGCTT                                          311
NEOGEN4.MCOI.NTD(42)           TGTCTGCTTCGATTATTGATTCGTTGTTTCATGATACTTGATTTGTTGTT 400
NEOGEN2.MCOI.NTD(13)           TATCTGCTTCAGTGATTGATTCTTTGTTTCATGATACTTGATTTGTTGTT 400
NEOGEN3.I.MCOI.NTD(3)          TATCTGCTTCGGTAGTTGATTCGTTATTTCATGATACTTGGTTTGTTGTT 400
NEOGEN3.II.MCOI.NTD(1)         TATCTGCTTCGGTGGTTGATTCTTTATTTCATGATACTTGGTTTGTTGTT 400
NEOGEN6.MCOI.NTD(1)            TATCTGCTTCTATTATTGATTCTTTG                         376
NEOGEN19.MCOI.NTD(2)                                                              324
NEOGEN18.MCOI.NTD(4)                                                              324
NEOGEN5.I.MCOI.NTD(3)          TATCTGCTTCGGTGGTTGATTCATTGTTTCATGATACTTGGTTTGTTGTT 400
NEOGEN5.II.MCOI.NTD(8)         TATCTGCTTCGGTGGTTGATTCATTGTTTCATGATACTTGATTTGTTGTT 400
NEOGEN9.I.MCOI.NTD(9)          TGTCTTCGTCTGTTTTAGATTCGTTATTTCATGATACTTGATTTGTTGTT 400
NEOGEN9.II.MCOI.NTD(2)         TGTCTTCGTCTGTTTTAGATTCATTATTTCATGATACTTGATTTGTTGTT 400
NEOGEN11.MCOI.NTD(8)           TGTCTTCGTCTGTTTTAGATTCGTTGTTTCATGATACTTGATTTGTTGTT 400
NEOGEN13.I.MCOI.NTD(2)         TGTCTGCTTCAGTACTAGATT                              371
NEOGEN13.II.MCOI.NTD(1)        TGTCTGCTTCAGTGCTAGATT                              371
NEOGEN14.I.MCOI.NTD(2)         TATCTTCTTCTG                                       362
NEOGEN14.II.MCOI.NTD(3)        TATCTTCTTCTG                                       362
NEOGEN10.MCOI.NTD(11)          TGTCTGCTTCTGTTTTGGATTCATTATTTCATGATACTTGGTTTGTTGTT 400
NEOGEN15.I.MCOI.NTD(1)         TGTCTGCTTCTGTATTAGATTCATTGTTTCATGATACTTGGTTTGTTGTT 400
NEOGEN15.II.MCOI.NTD(3)        TGTCTGCTTCTGTATTAGATTCATTGTTTCATGATACTTGGTTTGTTGTT 400
NEOGEN12.MCOI.NTD(4)           TATCTGCTTCTGTATTAGATTCTTTATTTCATGATACATGGTTTGTTGTC 400
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Figure 3-12. Continued.

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NEOGEN8.I.MCOI.NTD(6)          GC                 367
NEOGEN8.II.MCOI.NTD(1)         GC                 367
NEOGEN8.III.MCOI.NTD(1)        GC                 367
NEOGEN1.MCOI.NTD(18)           GCTCATTTTCATTATG 416
NEOGEN17.MCOI.NTD(1)           GCTCATTTTCATTATG 375
NEOGEN7.MCOI.NTD(36)           GCTCATTTTCATTATG 416
NEOGEN16.MCOI.NTD(3)                              311
NEOGEN4.MCOI.NTD(42)           GCTCATTTTCATTATG 416
NEOGEN2.MCOI.NTD(13)           GCTCATTTTCATTATG 416
NEOGEN3.I.MCOI.NTD(3)          GCTCATTTTCATTATG 416
NEOGEN3.II.MCOI.NTD(1)         GCTCATTTTCATTATG 416
NEOGEN6.MCOI.NTD(1)                               376
NEOGEN19.MCOI.NTD(2)                              324
NEOGEN18.MCOI.NTD(4)                              324
NEOGEN5.I.MCOI.NTD(3)          GCTCATTTTCATTATG 416
NEOGEN5.II.MCOI.NTD(8)         GCTCATTTTCATTATG 416
NEOGEN9.I.MCOI.NTD(9)          GCTCATTTTCATTATG 416
NEOGEN9.II.MCOI.NTD(2)         GCTCATTTTCATTATG 416
NEOGEN11.MCOI.NTD(8)           GCTCATTTTCATTATG 416
NEOGEN13.I.MCOI.NTD(2)                            371
NEOGEN13.II.MCOI.NTD(1)                           371
NEOGEN14.I.MCOI.NTD(2)                            362
NEOGEN14.II.MCOI.NTD(3)                           362
NEOGEN10.MCOI.NTD(11)          GCTCATTTTCATTATG 416
NEOGEN15.I.MCOI.NTD(1)         GCTCATTTTCATTATG 416
NEOGEN15.II.MCOI.NTD(3)        GCTCATTTTCATTATG 416
NEOGEN12.MCOI.NTD(4)           GCTCATTTTCATTATG 416
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Figure 3-12. Continued.

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NEOGEN18.MCOI.AA        VYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAIVCLGSMVWA 47
NEOGEN19.MCOI.AA        VYVLILPGFGMVSYICMTLSNSESLFGYFGLVCAMGAIVCLGSMVWA 47
NEOGEN3.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN17.MCOI.AA                      ICMTLSNNESLFGYFGLVCAMGAIVCLGSMVWA 33
NEOGEN7.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAIVCLGSMVWA 50
NEOGEN4.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAIVCLGSMVWA 50
NEOGEN6.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN1.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNSESLFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN5.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN2.MCOI.AA      HPEVYVLILPGFGMVSHICMTLSNNESLFGYFGLVCAMGAIVCLGSMVWA 50
NEOGEN8.MCOI.AA      HPEVYVLILPGFGMVSHMCMTLSNNESMFGYFGLVCAMGAIVCLGSMVWA 50
NEOGEN9.MCOI.AA      HPEVYVLMIPGFGMVSHMCMVLSNSESVFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN11.MCOI.AA     HPEVYVLMIPGFGMVSHMCMVLSNSESVFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN14.I.MCOI.AA   HPEVYVLILPGFGMVSHICMVLSNNESVFGYFGLVCAMGAMVCLGSIVWA 50
NEOGEN14.II.MCOI.AA  HPEVYVLILPGFGMISHICMVLSNNESVFGYFGLVCAMGAMVCLGSIVWA 50
NEOGEN10.MCOI.AA     HPEVYVLILPGFGMVSHMCMVLSNNESVFGYFGLVCAMGAMVCLGSMVWA 50
NEOGEN12.MCOI.AA     HPEVYVLILPGFGMVSHMCMVLSNSECVFGYFGLVCAMGSIVCLGSVVWA 50
NEOGEN13.MCOI.AA     HPEVYVLILPGFGMVSHMCMVLSNNESVFGYFGLVCAMGAIVCLGSMVWA 50
NEOGEN15.MCOI.AA     HPEVYVLILPGFGMVSHMCMTLSNSESVFGYFGLVCAMGAMVCLGSMVWA 50
                                        *.***.*.:***********::*****:***

NEOGEN18.MCOI.AA     HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLIDPVV 97
NEOGEN19.MCOI.AA     HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 97
NEOGEN3.MCOI.AA      HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN17.MCOI.AA     HHMFVVGMDVKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 83
NEOGEN7.MCOI.AA      HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN4.MCOI.AA      HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN6.MCOI.AA      HHMFVVGMDIKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN1.MCOI.AA      HHMFVVGMDMKTAVFFSSVTMMIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN5.MCOI.AA      HHMFVVGMDMKTAVFFSSVTMIIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN2.MCOI.AA      HHMFVVGMDMKTAVFFSSVTMIIGIPTGMKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN8.MCOI.AA      HHMFVVGMDMKTAVFFSSVTMIIGIPTGIKVFSWLYMLGSSYVRLVDPVV 100
NEOGEN9.MCOI.AA      HHMFVIGMDMKTAVFFSSVTMIIGVPTGMKVFSWLYMLGGSNVRLFDPVV 100
NEOGEN11.MCOI.AA     HHMFVVGMDMKTAVFFSSVTMIIGVPTGMKVFSWLYMLGGSNVRLFDPVV 100
NEOGEN14.I.MCOI.AA   HHMFVVGMDMKTAVFFSSVTMIMGVPTGMKVFSWLYMLGGSGVRLFDPVV 100
NEOGEN14.II.MCOI.AA  HHMFVVGMDMKTAVFFSSVTMIMGVPTGMKVFSWLYMLGGSGVRLFDPVV 100
NEOGEN10.MCOI.AA     HHMFVIGMDMKTAVFFSSVTMIIGMPTGIKVFSWLYMLGNSGVRLLDPVV 100
NEOGEN12.MCOI.AA     HHMFVIGMDLKTAVFFSSVTMIMGVPTGIKVFSWLYMLGGSGVRVYDPVV 100
NEOGEN13.MCOI.AA     HHMFVVGMDMKTAVFFSSVTMIIGIPTGMKVFSWLYMLGGSGVRLYDPVV 100
NEOGEN15.MCOI.AA     HHMFVVGMDVKTAVFFSSVTMMIGMPTGMKVFSWLFMLGSSNIRVYDPMV 100
                     *****:*** ***********::*:***:******:***.* :*: **:*

NEOGEN18.MCOI.AA     WWIMGFIFLF                                 107
NEOGEN19.MCOI.AA     WWIMGFIFLF                                 107
NEOGEN3.MCOI.AA      WWIMGFIFLFTVGGVTGIVLSASVVDSLFHDTWFVVAHFHYV 142
NEOGEN17.MCOI.AA     WWIVGFIFLFTMGGVTGIVLSASVIDSLFHDTWFVVAHFHY  124
NEOGEN7.MCOI.AA      WWIMGFIFLFTVGGVTGIVLSASVIDSLFHDTWFVVAHFHYV 142
NEOGEN4.MCOI.AA      WWIMGFIFLFTVGGVTGIVLSASIIDSLFHDTWFVVAHFHYV 142
NEOGEN6.MCOI.AA      WWVMGFIFLFTMGGVTGIVLSASIIDSLFHDTWFVVAHFHYV 142
NEOGEN1.MCOI.AA      WWIVGFIFLFTMGGVTGIVLSASVIDSLFHDTWFVVAHFHYV 142
NEOGEN5.MCOI.AA      WWIMGFIFLFTMGGVTGIVLSASVVDSLFHDTWFVVAHFHYV 142
NEOGEN2.MCOI.AA      WWIMGFIFLFTMGGVTGIVLSASVIDSLFHDTWFVVAHFHYV 142
NEOGEN8.MCOI.AA      WWMMGFIFLFTVGGVTGMVLSASVVDSLFHDTWFVVAHFHYV 142
NEOGEN9.MCOI.AA      WWIVGFMFLFTVGGVTGMVLSSSVLDSLFHDTWFVVAHFHYV 142
NEOGEN11.MCOI.AA     WWIVGFMFLFTVGGVTGMVLSSSVLDSLFHDTWFVVAHFHYV 142
NEOGEN14.I.MCOI.AA   WWVIGFMFLFVVGGVTGVVLSSSVLDSLFHDTWFVVAHFHYV 142
NEOGEN14.II.MCOI.AA  WWVIGFMFLFVVGGVTGIVLSSSVLDSLFHDTWFVVAHFHYV 142
NEOGEN10.MCOI.AA     WWVVGFMFLFTIGGVTGMVLSASVLDSLFHDTWFVVAHFHYV 142
NEOGEN12.MCOI.AA     WWILGFVFLFTMGGVTGMVLSASVLDSLFHDTWFVVAHFHYV 142
NEOGEN13.MCOI.AA     WWVIGFMFLFTIGGVTGMVLSASVLDSLFHDTWFVVAHFHYV 142
NEOGEN15.MCOI.AA     WWMIGFVFLFTIGGITGVVLSASVLDSLFHDTWFVVAHFHYV 142
                     **  ** ***  ** **  ** *  ****************
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Figure 3-13. Alignment of partial predicted amino acid sequences of the mitochondrial cytochrom oxidase I gene for *Neospirorchis* specimens. Shaded positions are differences between like genotypes based on predicted amino acid (mitochondrial cytochrome oxidase I) sequence and ribosomal ITS2 sequence.

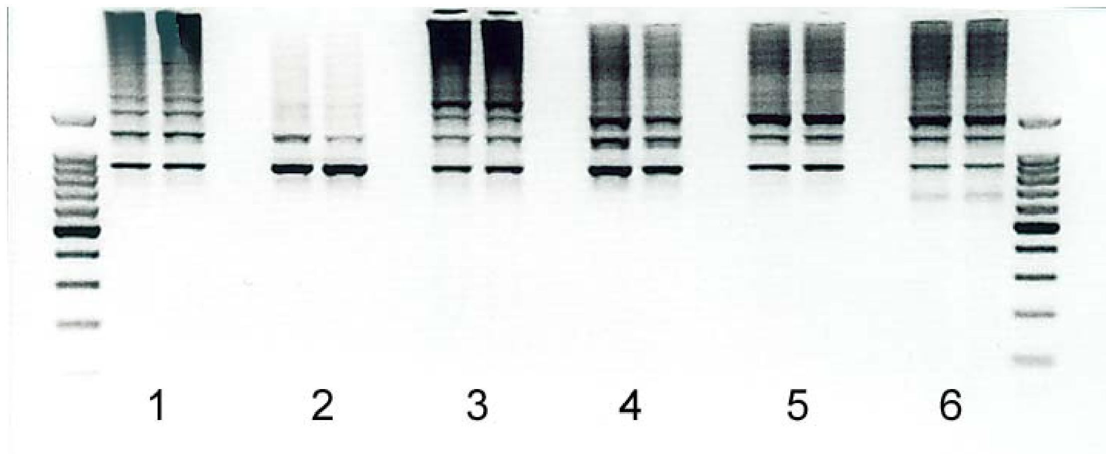

Figure 3-14. Gel electrophoresis of products obtained by PCR amplification of the ITS1. A forward primer within the 3' end of the 18s and a reverse primer within the ITS2 produced amplicons of multiple sizes. Side-by-side lanes represent replicates of the same sample. Differences in band sizes are due to variable numbers of tandem repeats. With the exception of some variation in intensity, band patterns are nearly identical between like genotypes. Samples 1 and 2 = Neogen-9, Sample3 = Neogen-10, Sample 4, 5, and 6 = Neogen-11.

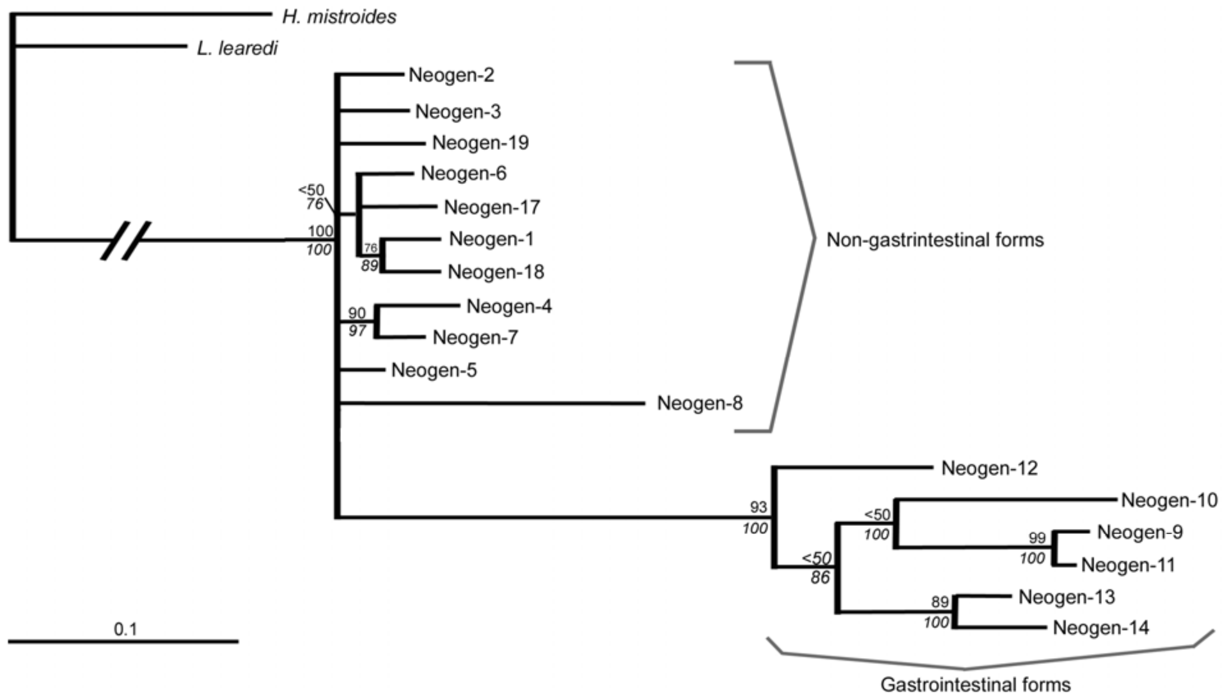

Figure 3-15. Bayesian phylogenetic tree of 270 to 306 nucleotides of the internal transcribed spacer region 2 of the ribosomal gene of *Neospirorchis* based on T-Coffee alignment. Bayesian posterior probabilities (italics) as percentages are given below branch nodes. Only branches with a probability of greater than 60% are shown. The ML bootstrap values are based on 200 re-samplings and are given above each node. Other marine spirorchiids, *H. mistroides* and *L. learedi*, were used as an outgroup. Brackets indicate the anatomic locations from which specimens within groups were collected.

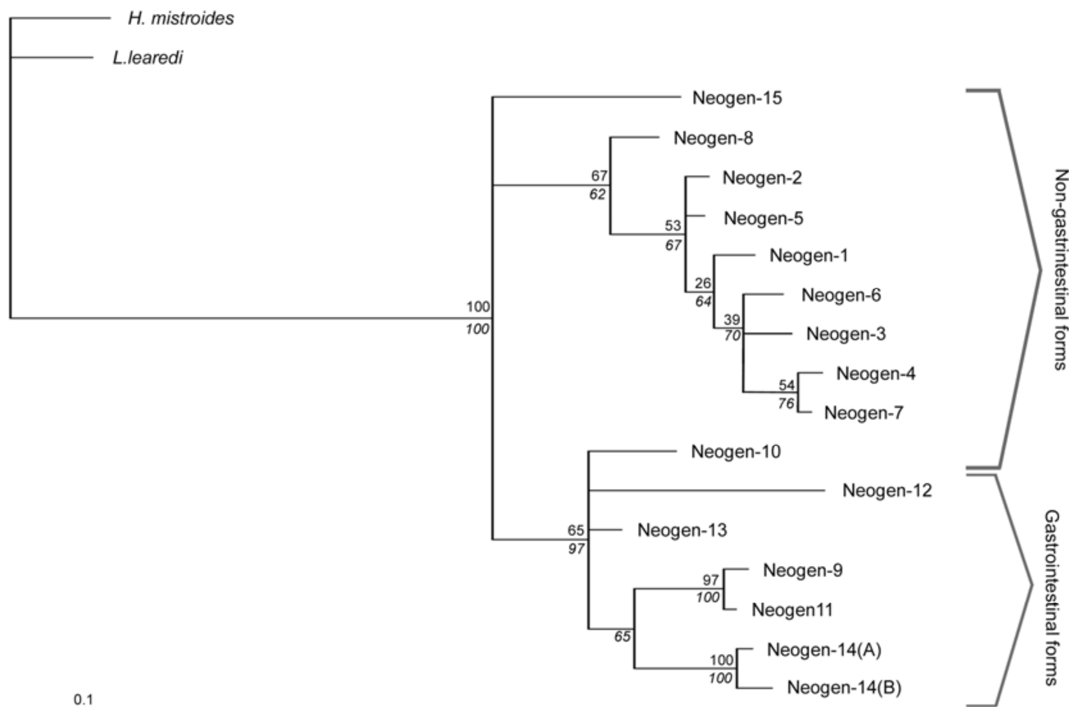

Figure 3-16. Bayesian phylogenetic tree of 142 partial amino acid sequence of the mitochondrial cytochrome oxidase I gene of *Neospirorchis* based on ClustalW alignment. Bayesian posterior probabilities (italics) as percentages are given below branch nodes. Only branches with a probability of greater than 60% are shown. The ML bootstrap values are based on 100 re-samplings and are given above each node. Other marine spirorchiids, *H. mistroides* and *L. learedi*, were used as an outgroup. The anatomic locations from which specimens within groups were collected are indicated by brackets.

Table 3-1. *Hapalotrema*, *Learedius learedi*, and *Carettacola bipora* specimens from by locality, anatomic location, host species, sample number, and gene sequence obtained.

Host ID	Genus & species	Locality	Location	Host	No.	ITS2	MCOI
Hawaii 1-10	*H. postorchis*	Hawaii	Unknown	CM	10	X	X
H.post.CR1-4	*H. postorchis*	Caribbean	Unknown	CM	4	X	
05-64	*H. postorchis*	Florida	Left aorta	CM	7	X	X
05-26	*H. pambanensis*	Florida	Heart	CC	1	X	X
06-72	*H. pambanensis*	Florida	Heart	CC	2	X	X
07-51	*H. pambanensis*	Florida	Liver	CC	2	X	X
07-51	*H. pambanensis*	Florida	Left aorta	CC	1	X	X
06-51	*H. pambanensis*	Florida	Heart	CC	1	X	X
05-45	*H. mistroides*	Florida	Left aorta	CC	2	X	X
05-45	*H. mistroides*	Florida	Mes. artery	CC	1	X	X
05-51	*H. mistroides*	Florida	Left aorta	CC	1	X	X
05-57	*H. mistroides*	Florida	Left aorta	CC	1	X	X
05-83	*H. mistroides*	Florida	Left aorta	CC	1	X	X
06-07	*H. mistroides*	Florida	Left aorta	CC	1	X	X
06-18	*H. mistroides*	Florida	Mes. artery	CC	1	X	X
07-13	*H. mistroides*	Florida	Mes. artery	CC	2	X	X
07-51	*H. mistroides*	Florida	Left aorta	CC	1	X	X
06-32	*Hap.* novel sp.	Florida	Liver	CC	4	X	X
07-45	*L. learedi*	Florida	Heart	CM	10	X	X
05-64	*L. learedi*	Florida	Heart	CM	4	X	X
08-96	*L. learedi*	Florida	Heart	CM	10	X	X
Hawaii1-10	*L. learedi*	Hawaii	Heart	CM	10	X	X
CTF1-10	*L. learedi*	Caribbean	Heart	CM	10	X	X
L. lear.CR1	*L. learedi*	Caribbean	Heart	CM	1	X	
05-51	*C. bipora*	Florida	Liver	CC	1	X	
05-58	*C. bipora*	Florida	Liver	CC	1	X	
06-12	*C. bipora*	Florida	Multiple	CC	3	X	X

Table 3-2. Internal transcribed spacer 2 pairwise distances between *Hapalotrema*, *Learedius*, and *Carettacola* specimens.

		1	2	3	4	5	6	7
1	*H. post*-A/C							
2	*H. post* -HW	0.026						
3	*H. mistroides*	0.053	0.053					
4	*H. pambanensis*	0.154	0.154	0.165				
5	*Hap*. novel sp.	0.139	0.147	0.143	0.158			
6	*L. learedi* (varA)	0.143	0.135	0.147	0.113	0.128		
7	*L. learedi* (varB)	0.147	0.139	0.147	0.113	0.128	0.008	
8	*C. bipora*	0.335	0.327	0.338	0.323	0.316	0.327	0.335

Shaded cells reflect pairwise comparisons between species of different locality (*H. postorchis*) or cases in which variation in the ITS2 was observed (*L.learedi*).

Table 3-3. Mitochondrial cytochrome oxidase I gene and pairwise distances (nucleotide) between *Hapalotrema*, *Learedius*, and *Carettacola* specimens.

		1	2	3	4	5	6	7	8	9
1	*H. post*-A/C[a]									
2	*H. post* -HW	0.082								
3	*H. mistroides*[a]	0.106	0.106							
4	*H. pambanensis*[a]	0.170	0.146	0.169						
5	*Hap.* novel sp.[a]	0.169	0.158	0.168	0.168					
6	*L. learedi* (FL.varA)[a]	0.142	0.157	0.136	0.162	0.171				
7	*L. learedi* (FL.varB)[a]	0.149	0.141	0.140	0.149	0.174	0.077			
8	*L. learedi* (HW.varA)[a]	0.149	0.134	0.142	0.156	0.167	0.076	0.029		
9	*L. learedi* (HW.varB)	0.149	0.150	0.144	0.152	0.174	0.073	0.046	0.045	
10	*C. bipora*	0.196	0.202	0.172	0.222	0.207	0.186	0.179	0.182	0.193

[a]Pairwise distances reflect an average of p-distances observed within the each designated type due to sequence variation. Shaded cells reflect pairwise comparisons between species of different locality (*H. postorchis*) or cases in which variation in the ITS2 was observed (*L.learedi*).

Table 3-4. *Neospirorchis* specimens by host, anatomic location, sample type, gene sequence obtained, and genotype.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
Nervous system						
N05-31	CC	Meninges	Eggs	X	X	NEOGEN-1
N05-44	CC	Meninges	Adult	X	X	NEOGEN-1
N05-58	CC	Meninges	Eggs	X		NEOGEN-1
N05-81	CC	Meninges	Eggs	X	X	NEOGEN-1
N05-83	CC	Meninges	Eggs	X	X	NEOGEN-1
N05-89	CC	Meninges	Eggs	X	X	NEOGEN-1
N06-07	CC	Meninges	Adult	X	X	NEOGEN-1
N06-12	CC	Meninges	Adult	X	X	NEOGEN-1
N06-20	CC	Meninges	Adult	X	X	NEOGEN-1
N06-51	CC	Meninges	Adult	X		NEOGEN-1
N06-56	CC	Meninges	Adult	X	X	NEOGEN-1
N07-46	CC	Meninges	Adult	X	X	NEOGEN-1
CXO2006112401	CC	Meninges	Adult	X	X	NEOGEN-1
NME2006090301	CC	Meninges	Adult	X	X	NEOGEN-1
NMY2006101801	CC	Meninges	Eggs	X		NEOGEN-1
T5;6/20/06	CC	Meninges	Adult	X	X	NEOGEN-1
T6;6/20/06	CC	Meninges	Adult	X		NEOGEN-1
T7;6/20/06	CC	Meninges	Adult	X	X	NEOGEN-1
T8;6/20/06	CC	Meninges	Adult	X	X	NEOGEN-1
N05-07	CC	Meninges	Adult	X	X	NEOGEN-2
N05-45 (A)	CC	Meninges	Adults & Eggs	X	X	NEOGEN-2
N05-45 (B)	CC	Meninges	Adult	X		NEOGEN-2
N05-45 (C)	CC	Meninges	Eggs	X		NEOGEN-2
N05-57	CC	Meninges	Adults & Eggs	X	X	NEOGEN-2
N05-63	CC	Meninges	Eggs	X	X	NEOGEN-2
N06-18	CC	Meninges	Adult	X	X	NEOGEN-2
N06-37	CC	Meninges	Adult	X	X	NEOGEN-2
N06-44	CC	Meninges	Adult	X	X	NEOGEN-2
N06-50	CC	Meninges	Adult	X	X	NEOGEN-2
N06-53	CC	Meninges	Adult	X	X	NEOGEN-2
T1;11/15/05	CC	Meninges	Adult	X	X	NEOGEN-2
T1;6/20/06	CC	Meninges	Adult	X	X	NEOGEN-2
T1;7/19/05	CC	Meninges	Adult	X	X	NEOGEN-2
T2;11/15/05	CC	Meninges	Adult	X		NEOGEN-2
T4;11/15/05	CC	Meninges	Adult	X	X	NEOGEN-2
N05-59	CM	Meninges	Adult	X	X	NEOGEN-3
N05-64	CM	Meninges	Adult s & Eggs	X	X	NEOGEN-3
N07-45	CM	Meninges	Adult	X	X	NEOGEN-3
T9;6/20/06	CM	Meninges	Adult	X	X	NEOGEN-3
BAO2006092101	CM	Meninges	Eggs	X	X	NEOGEN-17
N06-68	CC	Meninges	Adult	X		NEOGEN-5

Table 3-4. Continued.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
N05-91	CC	Meninges	Eggs	X		NEOGEN-8
N05-10	CC	Meninges[a]	Eggs	X	X	Mixed
N05-26	CC	Meninges[a]	Eggs	X		NEOGEN-4
N05-06		Meninges[a]	Eggs	X		NEOGEN-8
MCK2006100801	LK	Olfact. N	Adult	X	X	NEOGEN-1
AMF2006091101	CC	Optic n.	Adult	X	X	NEOGEN-6
Endocrine organs						
GST0803	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0814	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0820	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0821	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0824	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0825	CM	Thyroid	Adult	X	X	NEOGEN-7
GST0825	CM	Thyroid	Eggs	X	X	NEOGEN-7
GST0828	CM	Thyroid	Eggs	X	X	NEOGEN-7
GST0831	CM	Thyroid	Adult	X	X	NEOGEN-7
N05-06	CC	Thyroid	Eggs	X	X	NEOGEN-4
N05-26	CC	Thyroid	Eggs	X	X	NEOGEN-4
N05-45	CC	Thyroid	Adults & Eggs	X		NEOGEN-4
N05-46	CC	Thyroid	Adult	X	X	NEOGEN-4
N05-51	CC	Thyroid	Adult	X	X	NEOGEN-4
N05-57	CC	Thyroid	Eggs	X	X	NEOGEN-4
N06-13	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-13	CC	Thyroid	Eggs	X		NEOGEN-4
N06-18	CC	Thyroid	Eggs	X	X	NEOGEN-4
N06-32	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-33	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-37	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-44	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-68	CC	Thyroid	Adult	X	X	NEOGEN-4
N06-69	CC	Thyroid	Eggs	X	X	NEOGEN-4
N06-81	CC	Thyroid	Eggs	X	X	Mixed
N07-13	CC	Thyroid	Adult	X	X	NEOGEN-4
T1;7/19/05	CC	Thyroid	Adult	X	X	NEOGEN-4
T1;7/19/05	CC	Thyroid	Eggs	X		NEOGEN-4
T2;7/19/05	CC	Thyroid	Eggs	X	X	NEOGEN-4
T3;6/20/06	CC	Thyroid	Eggs	X	X	NEOGEN-4
T4;11/15/05(A)	CC	Thyroid	Eggs	X	X	NEOGEN-4
T4;11/15/05(B)	CC	Thyroid	Eggs	X	X	NEOGEN-4
T5;11/15/05	CC	Thyroid	Adult	X	X	NEOGEN-4
T5;6/20/06	CC	Thyroid	Eggs	X	X	NEOGEN-4
T6;6/20/06	CC	Thyroid	Adult	X	X	NEOGEN-4
N05-10	CC	Thyroid	Adult	X		NEOGEN-5
N05-31	CC	Thyroid	Eggs	X	X	NEOGEN-5
N05-58	CC	Thyroid	Adult	X	X	NEOGEN-5
N05-88	CC	Thyroid	Adult	X	X	NEOGEN-5
N05-91	CC	Thyroid	Eggs	X	X	NEOGEN-5

Table 3-4. Continued.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
N06-12	CC	Thyroid	Adults & Eggs	X	X	NEOGEN-5
N06-63	LK	Thyroid	Adults & Eggs	X	X	NEOGEN-5
T1;11/15/05	CC	Thyroid	Adult	X	X	NEOGEN-5
T1;6/20/06	CC	Thyroid	Eggs	X		NEOGEN-5
T7;6/20/06	CC	Thyroid	Adult	X	X	NEOGEN-5
N05-83	CC	Thyroid	Eggs	X		NEOGEN-6
N05-48	CC	Thyroid	Eggs	X	X	NEOGEN-6(99)
N05-59	CM	Thyroid	Eggs	X	X	NEOGEN-7
N05-64	CM	Thyroid	Eggs	X	X	NEOGEN-7
N06-46	CM	Thyroid	Adult	X	X	NEOGEN-7
N06-89	CM	Thyroid	Eggs	X	X	NEOGEN-7
N05-07	CC	Adrenal	Adult	X	X	NEOGEN-4
N05-46	CC	Adrenal	Adult	X	X	NEOGEN-4
N06-13	CC	Adrenal	Adult	X	X	NEOGEN-4
N06-37	CC	Adrenal	Adult	X	X	NEOGEN-4
N06-56	CC	Adrenal	Adult	X	X	NEOGEN-4
T4;6/20/06	CC	Adrenal	Adult	X	X	NEOGEN-4
T5;6/20/06	CC	Adrenal	Adult	X	X	NEOGEN-4
N05-58	CC	Adrenal	Adult	X	X	NEOGEN-5
N06-12	CC	Adrenal	Adult	X	X	NEOGEN-5
T7;6/20/06	CC	Adrenal	Adult	X	X	NEOGEN-5
N05-49	CM	Adrenal	Eggs	X	X	NEOGEN-7
N06-53	CC	Pineal gland	Eggs	X	X	NEOGEN-4
N06-46	CM	Pineal gland	Eggs	X	X	NEOGEN-7
RAB2006070901	CM	Pineal gland	Adult	X	X	NEOGEN-7
T5;7/19/05	CM	Pineal gland	Eggs	X	X	NEOGEN-7
Thymus						
N05-07	CC	Thymus	Eggs	X	X	NEOGEN-4
N05-26	CC	Thymus	Eggs	X	X	NEOGEN-4
N05-46	CC	Thymus	Adult	X		NEOGEN-4
N05-51	CC	Thymus	Eggs	X	X	NEOGEN-4
N05-57	CC	Thymus	Eggs	X	X	NEOGEN-4
N06-13	CC	Thymus	Eggs	X	X	NEOGEN-4
N06-18	CC	Thymus	Adult	X	X	NEOGEN-4
N06-32	CC	Thymus	Adult	X	X	NEOGEN-4
N06-37	CC	Thymus	Eggs	X	X	NEOGEN-4
N06-53	CC	Thymus	Eggs	X	X	NEOGEN-4
N06-69	CC	Thymus	Eggs	X	X	NEOGEN-4
T3;6/20/06	CC	Thymus	Eggs	X	X	NEOGEN-4
N05-83	CC	Thymus	Eggs	X	X	NEOGEN-6
Heart & Major arteries						
N05-10 (A)	CC	Heart	Adult	X	X	NEOGEN-8
N05-10 (B)	CC	Heart	Adult	X	X	NEOGEN-8
N06-12	CC	Heart	Adult	X		NEOGEN-8
N06-20 (A)	CC	Heart	Adult	X	X	NEOGEN-8
N06-20 (B)	CC	Heart	Adult	X	X	NEOGEN-8

Table 3-4. Continued.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
N06-20 (C)	CC	Heart	Adult	X	X	NEOGEN-8
N06-51 (A)	CC	Heart	Adult	X	X	NEOGEN-8
N06-51 (B)	CC	Heart	Adult	X	X	NEOGEN-8
N07-51	CC	Heart	Adult	X		NEOGEN-8
N05-44	CC	Artery	Adult	X	X	NEOGEN-8
N06-12	CC	Artery	Adult	X		NEOGEN-8
Gastrointestinal tract						
GST0822	CM	Stomach	Eggs	X	X	NEOGEN-14
N06-06	CM	Stomach	Eggs	X	X	NEOGEN-13
N07-45	CM	Stomach	Eggs	X	X	NEOGEN-13
N06-21	CM	Stomach	Eggs	X	X	NEOGEN-14
N06-89	CM	Stomach	Eggs	X	X	NEOGEN-14
N07-65	CM	Stomach	Eggs	X	X	NEOGEN-14
GST0805	CM	Stomach[a]	Eggs	X	X	NEOGEN-18
GST0813	CM	Stomach[a]	Eggs	X	X	NEOGEN-7
GST0820	CM	Stomach[a]	Eggs	X	X	NEOGEN-7
GST0824	CM	Stomach[a]	Eggs	X	X	NEOGEN-7
GST0827	CM	Stomach[a]	Eggs	X	X	NEOGEN-7
N05-10	CC	Intestine	Adult	X	X	NEOGEN-9
N05-10 (A)	CC	Intestine	Eggs	X		NEOGEN-9
N05-10 (B)	CC	Intestine	Eggs	X		NEOGEN-9
N05-26	CC	Intestine	Adult	X	X	NEOGEN-9
N05-44	CC	Intestine	Adult	X	X	NEOGEN-9
N05-90	CC	Intestine	Adult	X	X	NEOGEN-9
N06-18	CC	Intestine	Adult	X	X	NEOGEN-9
N06-26	LK	Intestine	Eggs	X	X	NEOGEN-9
N06-51	CC	Intestine	Adult	X		NEOGEN-9
N06-53	CC	Intestine	Adult	X	X	NEOGEN-9
N06-67	CC	Intestine	Eggs	X	X	NEOGEN-9
N06-69	CC	Intestine	Eggs	X	X	NEOGEN-9
N06-72	CC	Intestine	Adult	X	X	NEOGEN-9
N07-13	CC	Intestine	Eggs	X		NEOGEN-9
T8;6/20/06	CC	Intestine	Adult	X	X	NEOGEN-9
N05-31	CC	Intestine	Adult	X	X	NEOGEN-10
N05-51	CC	Intestine	Adult	X	X	NEOGEN-10
N05-57	CC	Intestine	Adult	X	X	NEOGEN-10
N05-58	CC	Intestine	Adult	X	X	NEOGEN-10
N06-12	CC	Intestine	Eggs	X		NEOGEN-10
N06-13	CC	Intestine	Adult	X	X	NEOGEN-10
N06-33	CC	Intestine	Adult	X	X	NEOGEN-10
N06-51	CC	Intestine	Adult	X		NEOGEN-10
N06-53	CC	Intestine	Adult	X	X	NEOGEN-10
N06-68	CC	Intestine	Adult	X	X	NEOGEN-10
N06-81	CC	Intestine	Adult	X	X	NEOGEN-10
T2;6/20/06	CC	Intestine	Adult	X	X	NEOGEN-10
N05-26	CC	Intestine	Adult	X	X	NEOGEN-11
N05-30	CC	Intestine	Adult	X	X	NEOGEN-11

Table 3-4. Continued.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
N06-12	CC	Intestine	Adult	X		NEOGEN-11
N06-20	CC	Intestine	Eggs	X		NEOGEN-11
N06-32	CC	Intestine	Adult	X	X	NEOGEN-11
N06-32	CC	Intestine	Adult	X	X	NEOGEN-11
N06-37	CC	Intestine	Adult	X	X	NEOGEN-11
N06-44	CC	Intestine	Adult	X	X	NEOGEN-11
N06-81	CC	Intestine	Adult	X	X	NEOGEN-11
T4;11/15/05	CC	Intestine	Adult	X	X	NEOGEN-11
N05-30	CC	Intestine	Adult	X	X	NEOGEN-12
N05-51	CC	Intestine	Adult	X	X	NEOGEN-12
GST0810	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0813	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0814	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0816	CM	Intestine[a]	Eggs	X	X	NEOGEN-18
GST0821	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0823	CM	Intestine[a]	Eggs	X	X	NEOGEN-19
GST0825	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0828	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
GST0831	CM	Intestine[a]	Eggs	X	X	NEOGEN-7
N05-63	CC	Intestine[a]	Eggs	X		NEOGEN-5
N05-84	CC	Intestine[a]	Eggs	X		NEOGEN-5
N05-83	CC	Intestine[a]	Eggs	X		NEOGEN-6
N05-88	CC	Intestine[a]	Eggs	X		NEOGEN-6
N06-12	CC	Intestine[a]	Eggs		X	NEOGEN-10
N06-81	CC	Intestine[a]	Eggs		X	NEOGEN-9
N06-13	CC	Colon	Adult	X	X	NEOGEN-12
N06-81	CC	Colon	Adult	X	X	NEOGEN-12
N05-30	CC	Colon	Eggs	X	X	NEOGEN-13
N05-57	CC	Colon	Eggs	X	X	NEOGEN-13
N05-48	CC	Colon*	Eggs	X		NEOGEN-6(99)
N05-30	CC	Cloaca	Eggs	X		NEOGEN-11
N06-20	CC	Cloaca	Eggs	X	X	NEOGEN-15
N06-51	CC	Cloaca	Eggs	X	X	NEOGEN-15
N06-53	CC	Cloaca	Eggs	X	X	NEOGEN-15
N06-56	CC	Cloaca	Eggs	X	X	NEOGEN-15
Lungs						
N06-06	CM	Lung[a]	Eggs	X		NEOGEN-16
GST0802	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0803	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0805	CM	Lung[a]	Eggs	X	X	NEOGEN-18
GST0810	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0814	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0816	CM	Lung[a]	Eggs	X	X	NEOGEN-18
GST0820	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0821	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0823	CM	Lung[a]	Eggs	X	X	NEOGEN-19

Table 3-4. Continued.

SampleID	Host species	Location	Sample type	ITS2	MCOI	Genotype
GST0823	CM	Lung[a]	Eggs	X	X	NEOGEN-19
GST0824	CM	Lung[a]	Eggs	X	X	NEOGEN-7
GST0831	CM	Lung[a]	Eggs	X	X	NEOGEN-7
N06-37	CC	Lung[a]	Eggs	X		NEOGEN-6
T5;6/20/06	CC	Nasal mucosa[a]	Eggs	X	X	Mixed
Testes						
N06-13	CC	Testis	Adults & Eggs	X	X	NEOGEN-4
N06-68	CC	Testis	Eggs	X	X	NEOGEN-4
Liver						
N06-13 (A)	CC	Liver	Adult	X	X	NEOGEN-6
N06-13 (B)	CC	Liver	Adult	X	X	NEOGEN-6
Urinary bladder						
N06-56	CC	U. bladder	Eggs	X	X	NEOGEN-15
N05-45	CC	U. bladder[a]	Eggs	X	X	NEOGEN-4
Voucher specimens						
N. schisosomatoides (A)	CC	Voucher	Adult	X	X	NEOGEN-1
N. schisosomatoides (B)	CC	Voucher	Adult	X	X	NEOGEN-1

[a]Derived from eggs with a diffusely embolized pattern

Table 3-5. *Neospirorchis* genotypes by organ system and host species.

Genotype	Anatomic location(s)	Host species	No. parasite specimens	No. turtle hosts
NEOGEN-1	Meninges (n=20) Olfactory nerve (n=1)	CC (n=20) LK (n=1)	21	21
NEOGEN-2	Meninges (n=16)	CC	16	14
NEOGEN-3	Meninges (n=4)	CM	4	4
NEOGEN-4	Thyroid gland (n=25) Thymus (n=12) Adrenal gland (n=7) Testis (n=2) Pineal gland (n=1) Meninges (n=1)[a] Urinary bladder (n=1)[a]	CC	49	26
NEOGEN-5	Thyroid gland (n=10) Adrenal gland (n=3) Meninges (n=1) Intestine (2)*	CC (n=15) LK (n=1)	16	13
NEOGEN-6	Thyroid gland (n=2) Liver (n=2) Optic nerve (n=1) Thymus (n=1) Intestine (n=2)[a] Colon (n=1)[a] Lung (n=1)[a]	CC	10	6
NEOGEN-7	Thyroid gland (n=13) Pineal gland (n=3) Adrenal gland (n=1) Stomach (n=4)[a] Intestine (n=7)[a] Lung (n=8)[a]	CM	36	19
NEOGEN-8	Heart (n=9) Major artery (n=2) Meninges (n=3)[a]	CC	14	9
NEOGEN-9	Intestine (n=15)	CC (n=12) LK (n=1)	15	13
NEOGEN-10	Intestine (n=13)	CC	13	12
NEOGEN-11	Intestine (n=10) Cloaca (n=1)	CC	11	9
NEOGEN-12	Intestine (n=2) Colon (n=2)	CC	4	4
NEOGEN-13	Stomach (n=2) Colon (n=2)	CC CM	4	4
NEOGEN-14	Stomach (n=4)	CM	4	4
NEOGEN-15	Cloaca (n=4) Urinary bladder (n=1)	CC	5	4
NEOGEN-16	Lung (n=1)*	CM	1	1
NEOGEN-17	Meninges (n=1)	CM	1	1
NEOGEN-18	Lung (n=2)[a] Stomach (n=1)[a] Intestine (n=1)[a]	CM	4	2
NEOGEN-19	Lung (n=1)[a] Intestine (n=1)[a]	CM	2	1

CC = *Caretta caretta*; CM = *Chelonia mydas*; LK = *Lepidochelys kempi*

[a] Derived from eggs with a diffusely embolized pattern

ITS2

Table 3-6. Mitochondrial cytochrome oxidase I gene and internal transcribed spacer 2 pairwise distances (nucleotide) between *Neospirorchis* specimens.

Neogen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.039	0.052	0.045	0.045	0.039	0.039	0.071	0.136	0.143	0.136	0.143	0.156	0.143	0.240	0.032	0.032	0.052
2	0.091		0.013	0.039	0.019	0.039	0.019	0.039	0.117	0.136	0.117	0.123	0.136	0.123	0.234	0.032	0.032	0.019
3	0.082	0.085		0.045	0.032	0.052	0.032	0.052	0.123	0.149	0.123	0.130	0.136	0.123	0.234	0.032	0.045	0.019
4	0.091	0.072	0.080		0.045	0.058	0.032	0.071	0.136	0.162	0.136	0.143	0.162	0.149	0.240	0.045	0.052	0.039
5	0.078	0.062	0.045	0.072		0.045	0.026	0.058	0.123	0.156	0.123	0.130	0.143	0.130	0.240	0.039	0.039	0.039
6	0.058	0.098	0.082	0.080	0.069		0.039	0.065	0.143	0.149	0.143	0.156	0.162	0.149	0.247	0.032	0.032	0.052
7	0.098	0.065	0.078	0.062	0.083	0.091		0.058	0.136	0.149	0.136	0.130	0.156	0.143	0.240	0.032	0.032	0.026
8	0.094	0.081	0.117	0.095	0.103	0.105	0.106		0.130	0.136	0.130	0.143	0.123	0.110	0.227	0.071	0.071	0.058
9	0.123	0.141	0.147	0.156	0.127	0.138	0.156	0.141		0.091	0.013	0.091	0.097	0.097	0.169	0.143	0.149	0.130
10	0.145	0.149	0.156	0.174	0.141	0.141	0.167	0.164	0.116		0.091	0.091	0.117	0.130	0.162	0.156	0.156	0.149
11	0.120	0.138	0.149	0.152	0.134	0.138	0.141	0.138	0.040	0.116		0.091	0.097	0.097	0.169	0.143	0.149	0.130
12	0.203	0.188	0.203	0.203	0.197	0.188	0.207	0.203	0.178	0.196	0.178		0.117	0.130	0.188	0.149	0.149	0.130
13	0.134	0.141	0.141	0.138	0.125	0.127	0.138	0.145	0.109	0.127	0.101	0.188		0.026	0.188	0.156	0.169	0.143
14	0.136	0.145	0.154	0.154	0.127	0.114	0.165	0.159	0.114	0.134	0.121	0.185	0.109		0.201	0.143	0.156	0.130
15	0.129	0.161	0.152	0.168	0.154	0.132	0.161	0.165	0.138	0.163	0.136	0.183	0.178	0.176		0.247	0.260	0.227
16																	0.026	0.032
17	0.062	0.080	0.101	0.091	0.089	0.069	0.094	0.098	0.134	0.167	0.138	0.181	0.145	0.147	0.143			0.045
18	0.087	0.051	0.072	0.054	0.053	0.083	0.065	0.095	0.123	0.149	0.134	0.210	0.134	0.129	0.156		0.076	
19	0.101	0.087	0.072	0.094	0.074	0.087	0.094	0.132	0.149	0.167	0.159	0.196	0.145	0.165	0.139		0.098	0.072

Mitochondrial cytochrome oxidase I

*Shaded areas reflect distances of less than 0.1

CHAPTER 4
MOLECULAR DETECTION OF MARINE SPIRORCHIIDS IN INTERMEDIATE HOSTS AND APPLICATION IN LIFE CYCLE STUDIES

Introduction

Ten genera and more than twenty different species of spirorchiids have been described in sea turtles; however, all of the life cycles remain unknown. Knowledge of parasite life cycles is essential to the understanding of virtually every aspect of host-parasite interaction, including epidemiology of infections and influences on prevalence and disease in the host. Three major issues complicate discovery of spirorchiid life cycles: the diversity of habitats utilized by sea turtles, the diversity of gastropods within those habitats, and the probability of low prevalence among intermediate hosts. In addition, most parasites have aggregated or overdispersed distribution patterns within host populations (Rhode, 1993). Thus, concentrations of infected intermediate hosts can be missed during field sampling. There have been only two reported attempts to discover the life cycles of marine spirorchiids, both of which were unsuccessful (Greiner et al., 1980; Dailey et al., 1992)

Although the life cycles of marine spirorchiids are unknown, major characteristics can be reasonably inferred from the known life cycles of other blood flukes, including spirorchiids of freshwater turtles. Both schistosomes and freshwater spirorchiids utilize a single gastropod intermediate host and infect the definitive host by penetrating the skin or mucous membranes. None of the blood flukes studied thus far infect their vertebrate hosts by ingestion. Infectious cercariae are short-lived and are not powerful swimmers, thus the definitive and intermediate hosts likely must be in relatively close proximity for transmission to occur. In addition, there is data to support that infection by at least some marine spirorchiids occurs in near shore habitat. Infections have been documented in captive-reared turtles at a coastal rehabilitation facility in

Florida (L. Herbst, personal communication) and at captive-breeding facility on Grand Cayman Island (Greiner et al., 1980). Also, serological evidence of exposure has been observed in captive *C. mydas* in Hawaii that were transferred into coastal seawater ponds (Work et al., 2005). Lastly, spirorchiid infections have not been observed in necropsied pelagic phase *C. caretta* recovered in the Canary Islands, which are immature animals of the Atlantic population (Oros et al., 2005). Thus, it is hypothesized that infection occurs after recruitment into the neritic habitat based on these observations

Classic parasitology methods rely on monitoring individual snails for cercarial emergence and microdissection to detect trematode infections. These methods are time consuming, do not detect early infections, and require that investigators sort through the many digenean taxa that may infect gastropods. With no advance knowledge to focus surveys, the effort required to investigate life cycles in the marine system by classical methods is daunting in terms of the number of different marine gastropod species and the numbers of individuals that must be screened.

A molecular approach to spirorchiid life cycle investigation offers many potential advantages, including high throughput screening of large numbers of gastropods, greater sensitivity, and specificity. Furthermore, developing digeneans can be specifically identified using the appropriate comparative genetic data. The life cycle investigation aspect of these studies included the following objectives: 1) develop a PCR method for sensitive and specific detection of spirorchiid trematodes known to infect Florida sea turtles; 2) adaptation this method into a technique for screening gastropods using a laboratory model; 3) screen gastropods for spirorchiids at two facilities where captive *C. mydas* are known acquire infections; 4) examine

gastropods found in seagrass beds for spirorchiid and non-spirorchiid trematode parasites of wild sea turtles.

With regard to the fourth objective, our hypothesis that sea turtle parasite transmission, including infection by some spirorchiids, occurs in seagrass beds is based on documented infections at one coastal facility and ecological features of seagrass habitat. First, captive-hatched *C. mydas* were observed to become infected by *Neospirorchis* sp. at a rehabilitation facility in which the predominant gastropod habitat in the adjoining waters is seagrass, suggesting that the intermediate host may be a species found in seagrass beds. Second, seagrass beds are important foraging grounds for both *C. caretta* and *C. mydas*, are globally distributed, and are very common coastal habitat in tropical and subtropical zones. In addition, seagrass beds are dominated by a relatively small number of gastropod species in terms of biomass, and many of these species are found in high density (Frankovich and Zieman, 2005). Sea turtles spend time in reasonable proximity to these organisms while foraging, and thus could become infected. Furthermore, *C. mydas* maintain distinct grazing plots where they may repeatedly encounter the same gastropod populations. The density of many gastropod populations, the relatively shallow depths of most seagrass beds, and importance of these areas to sea turtles make this habitat a seemingly ideal location for parasite transmission.

The findings of this study include the following: a method for molecular screening of large numbers of gastropods for trematode infections, with many potential applications in trematode life cycle studies; tentative identification of a limpet species, *Fissurella nodosa*, as an intermediate host of the marine spirorchiid *Learedius learedi*; and evidence that *Modulus modulus*, a gastropod species commonly found in sea grass, is the intermediate host of one, possibly two, alimentary trematode parasites of *C. mydas*. These findings are the first significant

advance in the elucidation of the life cycles of trematode parasites of sea turtles and hopefully will provide the means for the necessary confirmatory studies and discovery efforts in other regions.

Methods and Materials

Gastropod DNA Extraction

Multiple methods of tissue lysis and DNA extraction were tested to devise a method by which DNA could be extracted from relatively large quantities of gastropod tissue. Albino *Pomacea bridgesii* were used to test extraction methods so that pigment would not interfere with spectrophotometry. The protocol that best fulfilled our requirements in terms of sample volume, available equipment, DNA yield, and purity began with removal of gastropods from the shell and separation of the hepatopancreas and gonad. Only these organs were included in the DNA extraction, with the exception very small gastropods (less than 3 mm in greatest dimension) in which all soft tissue was processed. Gastropod tissues were combined to a total wet weight of 1.5 grams. Wet weight rather than individual numbers defined methods so that the technique would be applicable to gastropods of different sizes. The complete tissue lysis and DNA extraction protocol is given in Table 4-1. The lysis buffer was modified from Winnepenninckx et al. (1993) (Table 4-2). Included in this protocol is an additional step to remove melanin, which is abundant in the hepatopancreas and co-purifies with DNA. Multiple methods, including serial dilutions of DNA, were tested and the best result, in terms of PCR amplification and signal strength, was obtained by using the QIAquik PCR purification kit (Qiagen) for removal of pigment prior to PCR.

Polymerase Chain Reaction Protocol for Detection of Trematode DNA

Detection of trematode DNA was performed using polymerase chain reaction (PCR) targeting the internal transcribed spacer 2 (ITS2) of the ribosomal gene. This target was selected

because the ribosomal gene is present in the genome as a series of numerous repeats and thus supports sensitive detection. In addition, there is sufficient variation in the ITS2 to specific amplification. Several primers were designed and tested using different primer combinations, magnesium concentrations, and reaction conditions. Serial dilutions of *Neospirorchis pricei* and *Hapalotrema mistroides* DNA were prepared for testing of sensitivity. The DNA extracted from a non-spirorchiid digenean, *Calycodes anthos*, initially was used to assess specificity. Given that the intended use of this protocol was life cycle discovery, it was anticipated that all amplicons would be sequenced, thus further addressing any issues of specificity.

Four primers ultimately were selected for use in detection studies. The forward primer SPIR1 (5'-GAGGGTCGGCTTATTATCTATCA-3') and outer reverse primer SPIR2 (5'-TCACATCTGATCCGAGGTCA-3') were consensus primers complementary to the 3' end of the 18s gene and 5'-end of the 28s gene, respectfully. These primers amplify the ITS2 of a diverse variety of digenean trematodes. Two spirorchiid-specific reverse primers were designed for use with SPIR1 that were complimentary to areas within the ITS2. The first reverse primer was NCS2 (5'-CATTGAGCCACGACAAGG-3'), which is complementary to the ITS2 of all *Neospirorchis* species and genotypes identified from Florida turtles to date. The second reverse primer, HLC4 (5'-GCAGCAACTCAACCTGRTAAACC-3'), was designed to amplify the ITS2 of *Hapalotrema* species known to infect Florida turtles (*H. mistroides*, *H. postorchis*, and *H. pambanensis*) and *Learedius learedi*. Initially, the SPIR1 and NSC2, and SPIR1 and HLC4 were used in two reactions for the detection of spirorchiids. In later field studies conducted on Grand Cayman Island, the Marquesas Keys, and Florida Bay, a hemi-nested application was adopted to detect other trematode infections, in addition to spirorchiids. This hemi-nested technique included initial amplification of trematode ITS2 using the SPIR1 and SPIR2, followed by two

reactions using SPIR1 and the two specific reverse primers, NSC2 and HLC4. The Taqman PCR kit (Qiagen) was used for all reactions, which were performed in a 20µl reaction volume according to standard protocol. The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid, Franklin, MA). Reaction conditions for the first reaction included initial denaturation at 95°C for 5 min, then 45 cycles of denaturation at 95°C for 60 s; annealing at 50°C for 45 s, and DNA extension at 72°C for 120 s, followed by a final extension step at 72°C for 10 min (Table 4-3) . Similar conditions were used for the second reactions, except a higher annealing temperature of 56°C was used (Table 4-3).

The PCR products were resolved in 1% agarose gels and direct sequencing identified all bands of interest. The expected amplicon size for the spirorchiid-specific primers was 180 to 225 base pairs. Amplicons produced by the conserved primers typically were between 300 and 500 base pairs. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, NJ) and analyzed on ABI 377 automated DNA sequencers at the University of Florida Center for Mammalian Genetics DNA Sequencing Facilities.

Detection of Spirorchiids in Gastropods

The threshold of detection for the gastropod DNA extraction and PCR technique was assessed by spiking *P. bridgesii* total extracts with known quantities of spirorchiid DNA. Detection was measured using embryonated eggs as biological units. Eggs were obtained from the tissues of dead stranded turtles and counted into a 1.5 ml tube using a dissecting microscope and microcapillary tubes. To insure rupture of eggs and complete DNA extraction, eggs were placed into 180 µl of deionized water, frozen at -80° C, thawed, and then sonicated for 1 minute. These steps were repeated three times, which resulted in obvious rupture of approximately 90% of the eggs. Therefore, a 10% error rate was considered in egg DNA extractions and 110 eggs

were included to obtain DNA from a target of 100. After sonication, DNA was extracted using the DNeasy kit. To obtain the desired volume needed to spike *P. bridgesii* extracts, the total elution volume was then divided by the number of eggs extracted (100). Using this technique, gastropod DNA was spiked with one to ten egg equivalents of spirorchiid DNA. For positive controls, one complete adult spirorchiid (*L. learedi* or *Neospirorchis* sp.) was combined with 1.5 gm of snail tissues and extracted using the gastropod protocol.

Field Studies: Gastropod Collections and Synopsis of Study Sites

Gastropods were collected from four study sites: 1) coastal habitat adjoining The Turtle Hospital (TH), Marathon Florida; 2) coastal habitat adjoining the Cayman Turtle Farm, Limited (CTFL), Grand Cayman Island, British West Indies; 3) the Marquesas Keys region (MK); and 4) Florida Bay (FB). The Marine Conservation Board (Grand Cayman), the Key West National Wildlife Refuge (Marquesas Keys), and the Everglades National Park (Florida Bay) provided permits for collections. All collections were performed by snorkeling or under SCUBA. Most of the collected gastropods were stored frozen at -80°C until extraction of DNA and PCR were performed. A small subset of gastropods was examined for trematode infection by microdissection. Preparation of gastropods and DNA extraction was performed in a separate laboratory from PCR. Positive controls included DNA from *H. mistroides* and *Neospirorchis* species (30 fg of template DNA). Any suspected positive results were re-examined by repeating the pigment removal step (QIAquick spin column) using an additional aliquot of the original DNA extract and PCR. As in the model system, all amplicons of interest were sequenced for confirmation. Sequences were then compared with spirorchiid ITS2 sequences of *Neospirorchis*, *Hapalotrema*, *Learedius*, and *Carettacola* ITS2 sequences obtained from Florida turtles and from a limited set of ITS2 sequences available from non-spirorchiid trematode parasites of sea turtles. In addition, sequences were compared to those in the databases of GenBank (National

Center for Biotechnology Information, Bethesda, MD), EMBL (Cambridge, UK), and the Data Bank of Japan (Mishima, Shiuoka, Japan) using BLASTN (Altschul et al., 1990).

The Turtle Hospital, Marathon Key, Florida

The Turtle Hospital is on the Florida Bay side (north shore) of Marathon Key (Figure 4-1). Much of the adjoining marine habitat is seagrass beds with a depth of 1 to 2 meters. Sea turtles are brought to the TH for rehabilitation and hospital tanks utilize a simple flow-through system whereby seawater is pumped from the adjacent shallow inshore area. As previously mentioned, infection of captive-hatched *C. mydas* by *Neospirorchis* sp. has been was documented at the TH (L. Herbst, personal communication), which supports that the intermediate host occurs in the local habitat.

Cayman Turtle Farm, Limited (CTFL), Grand Cayman Island, British West Indies

As of 2006, The CTFL has a population of approximately 10,000 *C. mydas*. The facility has a flow-through filtration system whereby large volumes of seawater are pumped into the tanks via an open intake channel and the unprocessed effluent is discharged approximately 215 meters from the intake area (Figure 4-2). The coastal habitat consists of a limestone shore (ironshore) and hard bottom that descends relatively rapidly to a mini-wall (15 to 18 meters deep) and then to a shear main wall. The ironstone shore includes abundant tide pools (Figure 4-3) and the subtidal zone is extensively pocketed by burrows of rock-boring urchins (*Echinometra* sp.) (Figure 4-4). The seafloor is relatively barren from the shore to the coral reef associated with mini-wall and main wall.

Marquesas Keys region

The Marquesas study site consisted of two general areas, a large *C. mydas* grazing area located 2-4 kilometers west of actual islands, and the shallower Mooney Harbor lagoon within the island group and near shore zone on the outer southwestern-most islands (Figure 4-5). The

grazing plots were identified by observing for grazing turtles, investigating lighter areas within seagrass beds visible from the surface, or by snorkeling (Figure 4-6). The typical grazing area was 3 to 4 m deep and tidal current were variably intense. The lagoon and near shore collection sites most often were less 1.5 m deep. Both *C. caretta* and smaller immature *C. mydas* are frequently observed in the lagoon and near shore areas.

Florida Bay, Everglades National Park

The Florida Bay sites were within the area surveyed during annual *C. caretta* captures conducted by the Florida Fish and Wildlife Commission (FWC) in partnership with the National Marine Fisheries Service (NMFS). Based on these studies, *C. caretta* are believed to relatively abundant in this part of Florida Bay. The collection area included Arsniker Basin, Rabbit Key Basin, and Twin Key Basin (Fig 4-7).

Field Studies: Ancillary Data

Cayman Turtle Farm, Limited

Prior to gastropod collections, an advance site visit was conducted in December 2005 to obtain current prevalence data from *C. mydas* harvested for human consumption. The heart and major vessels of 30 turtles were examined for the presence of *L. learedi*. All chambers were opened and the blood was washed into a #45 seive. Adults were observed using a dissecting microscope and counted. Fecal examinations were performed on all 30 turtles using a standard detergent/sedimentation technique. In addition, the mucosa of representative sections of the intestine was examined for 22 of the 30 turtles. Lastly, fecal examinations were performed on the single resident *C. caretta* on multiple occasions in 12/2005, 7/2007, and 4/2008.

During gastropod collections in October 2006 and July 2007, the seawater coming into the CTFL via the intake channel was sampled using a vertical tow plankton net (Wildco®) with a 154 µm mesh catch bucket. The net was suspended in middle of the channel approximately 0.3

m from the surface and initially was emptied every twelve hours. Filtrates were examined for cercariae using a dissecting microscope. To minimize the filtrate volume and improve the viability of captured microorganisms, filtrates also were examined at three or four hour intervals on multiple occasions. Following examination, the remaining filtrate was frozen and transported to the University of Florida where DNA was extracted using the gastropod protocol and screened for the presence of *L. learedi* by PCR.

Marquesas Keys

Free-floating fecal samples were obtained from foraging *C. mydas* during all four gastropod collecting trips to the Marquesas grazing area. Fecal samples were consistent with that of *C. mydas* based on appearance and frequent association with feeding turtles. Parasitological examinations were performed using standard floatation and sedimentation techniques.

Results

Method for Screening Gastropods for Spirorchiid Trematodes by PCR

By testing serial dilutions of spirorchiid DNA, the PCR protocol using the SPIR1/NSC2 and SPIR1/HLC4 primer combinations in two reactions was found to detect spirorchiid DNA in template quantities as low as a few femtograms, which equates to the level of a single copy. A magnesium concentration of 2.5 mM and the previously described reaction conditions produced the greatest sensitivity and did not detect *C. anthos*. Although there is inevitable error in this estimation of analytical sensitivity, this threshold of detection, although approximated, was decided to be suitable for use in the gastropod model.

The gastropod DNA extraction technique using albino *P. bridgesii* yielded between 1.85 to 1.87 mg of DNA from 1.5 grams of starting material. Spectrophotometric analyses of 100-fold dilutions of DNA yielded 260/280 ratios of 1.73 to 1.76, indicating removal of most

contaminants. Thus, the purity of the extracted DNA using this method was appropriate for use in PCR. Detection of *L. learedi* ITS2 was attempted using the primers SPIR1/HLC4 and several different quantities of template DNA (10, 50, 100, 500, and 1000 ng) from snail tissues that were extracted with a single adult *L. learedi*. Snail tissues spiked with the *L. learedi* yielded single bright bands using all template DNA quantities, whereas *P. bridgesii* tissues that were not spiked were PCR negative. Based on these results, 100 ng of was selecting as the amount of template DNA used in each PCR reaction. Further testing of *P. bridgesii* spiked with various amounts of DNA from embryonated eggs found that as little as one egg equivalent could be detected using the DNA extraction protocol and SPIR1/HLC4 and SPIR1/NCS2 primer pairs (Figure 4-8). Furthermore, a brighter amplicon was detected with the use of the Qiagen PCR clean-up kit to remove melanin from wild-type *P. bridgesii* DNA, as compared to simply diluting the DNA (Figure 4-8). This level of sensitivity is comparable the earliest possible stage of infection in gastropods. Given host tissues of infected gastropods often are severely effaced by developing stages of trematodes, this level of detection was deemed suitable for application in field studies. In addition, sensitivity was further increased when the consensus primers (SPIR1 and SPIR2) were used in an initial reaction, followed by hemi-nested application of SPIR1 and the specific reverse primers.

Field Studies

The Turtle Hospital, Marathon, Florida

Gastropod collections were performed at The Turtle Hospital (TH) in June and August 2005 and 2006. All of the species collected were common inhabitants of seagrass beds (Table 4-4). No evidence spirorchiid infection was found in any of the 2,100 gastropods examined.

Cayman Turtle Farm, Limited, Grand Cayman Island, British West Indies

In advance of the life cycle studies, the prevalence of *L. learedi* in examined harvested *C. mydas* was found to be very high. All 30 turtles examined had either adult *L. learedi* in the cardiovascular system (29/30) or embolized eggs visible in the enteric submucosa (20/22). Numbers of adult *L. learedi* ranged from one to 57, with an average of 12.3 per turtle; however, *L.learedi* eggs were observed in the feces of only 10% (3/30) of infected turtles. Similarly, egg numbers in examined submucosal samples also were very small. The single resident *C. caretta* at the facility had negative fecal results on all three instances (2005, 2006, and 2007) in which samples were collected.

Gastropods were collected at the CTFL during October 2006, July 2007, and April 2008. A list of collected species is given in Table 4-5. A variety of tidal, intertidal, and subtidal species were examined. Almost all gastropods were collected within 5 meters of the shoreline; however, the surveyed habitat extended from the tidal pools within the iron shore to the nearshore mini-wall to the reef system of the upper main wall with a depth of approximately 15 to 18 meters. A single night collection was performed, but did yield significantly different species from daytime collections, with the exception of two *Cowry* species.

A total of 3,883 gastropods from the CTFL were screened by PCR. A single positive PCR result was obtained from a limpet species, *Fissurella nodosa*, collected in July 2007 (Figure 4-9). Multiple amplicons from three separate PCR runs were sequenced and identified as *L. learedi* ITS2. The positive result was from a pooled sample of thirteen individual limpets. A total of 550 *F. nodosa* were examined, including 413 that were collected during April 2008 in efforts targeting this species after the original positive result was obtained (Figure 4-10). No additional positive results were detected. Cercarial development was observed in 2.3% (10/438) of *F. nodosa* examined by microdissection, which included 25 individuals in July 2007 and 413 from

April 2008. All of these cercariae were cotylomicrocercous and sequence obtained using the consensus trematode primers indicated greatest homology with an opocoelid species (82% identify), which is consistent with the observed cercarial morphology. No cercariae were observed that had morphology consistent with developing blood flukes.

Of the 528 gastropods of other species examined by microdissection, cercarial development was only observed in four (0.76%). Infected species included *Fissurella barbadensis*, *Diodora listeri*, *Nodolittorina dilatata*, and *Cenchritas muricatus*. None of these cercariae exhibited morphology consistent with spirorchiids. Additional trematode infections (unidentified taxa) were detected in *Nodilittorina augustior*, *Nodilittorina mespillum*, *Nerita peloronta*, and *Cerithium littoratum* by consensus PCR.

All plankton net filtrates tested by PCR were positive for *L. learedi*. No free-swimming cercariae were observed in the plankton net filtrates; however, the myriad of different organisms made examination for soft-bodied forms, such as cercariae, very difficult to detect. There is considerable mixing of facility effluent with seawater in the nearshore zone. The effluent plume occasionally was observed to flow back directly into the intake channel. Although plankton net collections were not performed during these times, the positive PCR result from intake filtrates may reflect a combination of parasite material in the effluent, as well as any cercariae being discharged from infected intermediate hosts.

Marquesas Keys

Four gastropod collecting trips were conducted in the Marquesas Keys region August and December 2007 and March and June 2008. A total of 45 fecal samples were collected from the area. Results of fecal examinations are given in Table 4-6. Some of these samples may be from the same turtles given the collection technique, thus these results should be regarded as indication of the species of parasites present and do not support any extrapolation regarding

frequency or prevalence. Ova of two spirorchiid genera, *Learedius* and *Neospirorchis*, were detected as were ova of the non-spirorchiid digenean genera *Rhytidodes*, *Deuterobaris*, *Polyangium*, and *Schizamphistomoides*. Some unidentified immature trematodes and ova also were observed.

The abundance of gastropods was relatively low in the grazing areas, which had a depth of 3 to 4 meters and periods of intense tidal currents. As a result of these conditions and lower abundance, proportionately more effort was expended in the collections from grazing plots despite the lower number of gastropods collected relative to Mooney Harbor and shallower near shore zones. Both *C. mydas* and *C. caretta* were frequently observed during collections. Of the 295 gastropods examined by microdissection (Table 4-7), developing trematodes were only observed in one snail (*Cerithium* species). These microdissections included the four individuals of *Patelloidea pustulata*, the only limpet species observed.

A list of gastropods collected and screened by PCR is shown in Table 4-8. None of the samples tested were positive by PCR for the presence of spirorchiids. Select amplicons produced by the first round PCR (consensus primers) were sequenced when present and interpretable sequence was obtained for 29 samples, which included pooled tissues of the species *Modulus modulus*, *Columbella mercatoria*, *Columbella rusticoides*, *Lithopoma americanum*, *Cerithium atratum*, and *Crepidula* species (Figure 4-11). Three different samples of *M. modulus* collected from Mooney Harbor yielded ITS2 sequence that was 100% homologous with *Angiodictyum parallelum*, an alimentary trematode found in *C. mydas*. In addition, another ITS2 sequence was obtained from a sample of *M. modulus*, which included individuals collected from a grazing plot. This second sequence shared 92% identity with an unidentified pronocephalid

species collected from the stomach of a *C. mydas*. None of the remaining sequences could be matched to specific digenean ITS sequences available in the public databases.

Florida Bay

Gastropods were collected from seagrass beds in Florida Bay in October 2007. A list of species collected and screened by PCR is given in Table 4-9. No evidence of spirorchiid infection was detected by PCR. As for the Marquesas samples, select amplicons were sequenced for bands obtained using consensus trematode primers. Interpretable sequence was obtained for twelve sample batches, that included *Columbella mercatoria*, *Columbella rusticoides*, *Tegula fasciata*, *Cerithium eburneum*, and *Lithopoma americanum*. None of the sequences matched or were similar to available digenean sequences from sea turtles. Of the specimens collected, 295 also were examined by microdissection (Table 4-7). Only one *Cerithium* species had cercarial development, the morphology of which was not consistent with a spirorchiid and ITS2 sequence matched that of trematodes detected in other *Cerithium*, including ceracaria recovered from a single specimen from the Marquesas region.

Discussion

The utility of the DNA extraction and PCR method developed for high throughput screening of gastropods was evidenced by successful detection of digenean infections in many of the species examined. Ideally, gastropods exposed to infectious miracidia with known time points of infection would used to define sensitivity; however, this was not possible given that none of the life cycles had been discovered for any digenean parasites of sea turtles. The use of a non-target gastropod species, a captive-propagated freshwater species in this study, spiked with parasite material was a useful surrogate for validating the technique. The threshold of detection was found to be equivalent to the earliest possible prepatent infection based on the ability to detect a single egg equivalent in 1.5 grams of host tissue. Although this measure of sensitivity is

only an approximation, it is more than adequate for applications in life cycle discovery. Naturally infected gastropods likely will include individuals in which host organs are extensively effaced by developing parasites; therefore, the amount of parasite material present will exceed that of an embryonated egg by many orders of magnitude. Furthermore, the ribosomal gene, including the ITS targeted by the PCR technique, is present in the genome as numerous tandem repeats. Thus, each parasite cell contains many copies for detection by PCR.

The detection *L. learedi* in the limpet *F. nodosa* is the first evidence of the identity of an intermediate host of a marine spirorchiid; however, confirmatory studies are necessary. Efforts to observe spirorchiid cercarial development in additional specimens to support the molecular findings were unsuccessful, despite the gross examination of over 400 individual *F. nodosa*. A total of 137 *F. nodosa* were screened in the initial collection that included the positive sample, thus prevalence in the collected limpets was as low as less than 1% (assuming only one limpet was positive in the pooled sample). This finding is not inconsistent with expected prevalence in intermediate host populations. Prevalence can be very low in intermediate host populations and result in high prevalence in definitive hosts due to high fecundity of asexual stages, i.e. abundant production of cercaria, as well as prolonged survival of infected intermediate hosts. Holliman (1971) reported a prevalence of 0.11% for the freshwater spirorchiid *Spirorchis parvus* and hypothesized that high cercarial output rather than abundance of infected snails maintains this spirorchiid life-cycle. Gastropod hosts of freshwater spirorchiids have been documented to produce as many as 100 cercariae per day and live as long as several months (Goodchild and Fried, 1963; Holliman, 1971; Peiper, 1953). Daily cercaria output in the hundreds has been documented in the intermediate hosts of some *Schistosoma* (Marquardt et al., 2000). Other investigators have documented seasonal variation in the prevalence of spirorchiids, including

periods where parasites were undetectable (Fernandez, 1991; Rosen et al., 1994). In addition, although the prevalence of *L. learedi* infection appears to be high in harvest-age *C. mydas* at the CTFL, which are between 3 and 4 years old, fecal egg numbers were observed to be very low in all turtles examined. Only ten percent of infected turtles had eggs detected in the feces, and eggs were consistently rare and difficult to find in positive samples. Therefore, *L. learedi* may be cycling at a relatively low level of abundance in terms of fecal output of eggs into in the system. Another consideration is that the distribution of infected *F. nodosa* extends or is more concentrated outside of the study area, which was limited to boundaries of the CTFL under the collection permit. The flow of the effluent discharge is highly variable and depends on the prevailing current. Thus, eggs of *L. learedi* may be broadly distributed within the coastal habitat outside of the CTFL.

The possibility of laboratory contamination as an explanation for the positive result is remote. No addition positive PCR results for *L. learedi* were detected in the other 13,729 gastropods (570 pooled samples) screened during this study. Furthermore, dissection of gastropods and DNA extraction were performed in a laboratory separate from the PCR laboratory, and *H. mistroides* DNA rather than *L. leraedi* was used as a positive PCR control to facilitate recognition of any contamination. Another possible, although improbable, scenario other than infection is that the positive *F. nodosa* ingested the eggs, but was not actually infected. *Fissurella nodosa* is an intertidal species and feeds on algae. It is plausible that eggs adhered to the algae may be ingested. The intensity of the positive PCR result, however, was much stronger than observed in the detection model studies when small quantities, such as egg equivalents, were introduced. Also, none of the other limpet species or other gastropod species that also feed on algae yielded a positive result. Our conclusion is that the positive PCR result

supports that *F. nodosa* is an intermediate host for *L. learedi,* although confirmatory studies are needed, especially given that our findings were limited to a single positive sample. Furthermore, limpet species should be included among the species examined in future life cycle discovery efforts.

Although no evidence of spirorchiid infection was found in any of the other study sites, we were able to demonstrate that *Modulus modulus*, one of the most common gastropod species found in seagrass beds, is the intermediate host for *Angiodictyum parallelum* (Loss 1901), a digenean trematode of the alimentary tract of *C. mydas*. This parasite was detected in three separate *M. modulus* samples from two different collecting trips and all originated from Mooney Harbor, which is frequented by *C. mydas*. In addition, *M. modulus* also may be a host for an as of yet genetically uncharacterized pronocephalid species. The digenean ITS2 sequence obtained from a sample of *M. modulus* collected from a grazing plot was highly similar (92%) to that of an unidentified pronocephalid collected from the stomach of a *C. mydas*. This degree of similarity suggests that the digenean detected in *M. modulus* is a closely related organism and very likely is a sea turtle parasite. The identity of the digenean may be discovered as the library of comparative genetic sequences available for sea turtle parasites is expanded.

Examination of fecal samples opportunistically collected from the Marquesas grazing area provided useful insight into the parasite species present in turtles foraging at the study site. Although none of the parasite genera detected could be specifically linked with those found in the gastropod samples, we were able to demonstrate that *C. mydas* foraging in the area were infected by at least four genera of alimentary trematodes and two spirorchiid genera. This parasite surveillance data may be useful to future life cycle studies in the Marquesas region.

Conclusions

Digenean trematodes are the most diverse and numerous endoparasites of sea turtles; however, there has been very little progress toward discovery of their life cycles. The implications of this missing information are that critical aspects of the host-parasite relationship, including epidemiology and factors relevant to turtle health and disease, are poorly understood. The results of this study provide a useful molecular tool for life cycle discovery studies with proven field application and the first evidence of the identity of the gastropod intermediate hosts of at least two trematode parasites of *C. mydas*. In addition, the discovery that *M. modulus* serves as an intermediate host for *A. parallelum*, and possibly a pronocephalid species, supports the hypothesis that seagrass beds are a suitable habitat for transmission of trematode parasites of sea turtles. These findings may be used to guide the necessary follow-up studies and to overcome many of the seemingly overwhelming difficulties of life cycle discovery in the marine environment.

Figure 4-1. Marine system adjoining The Turtle Hospital, Marathon, Florida. The predominant gastropod habitat at this site is seagrass beds (*Thalassia testudinum*) (inset).

Figure 4-2. Satellite image of the Cayman Turtle Farm, Limited, Grand Cayman Island. The intake and effluent channels are labeled and are approximately 215 meters apart. The side-by-side round and square structures are tanks used for rearing *C. mydas*.

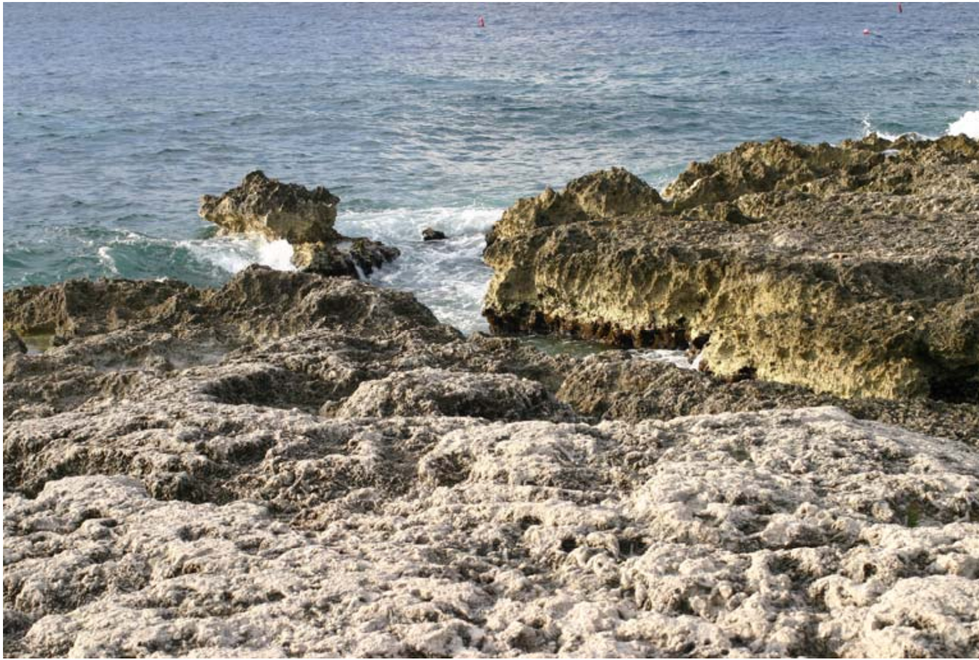

Figure 4-3. The limestone shore (ironshore) adjoining the Cayman Turtle Farm, Limited. Several large tide pools are visible.

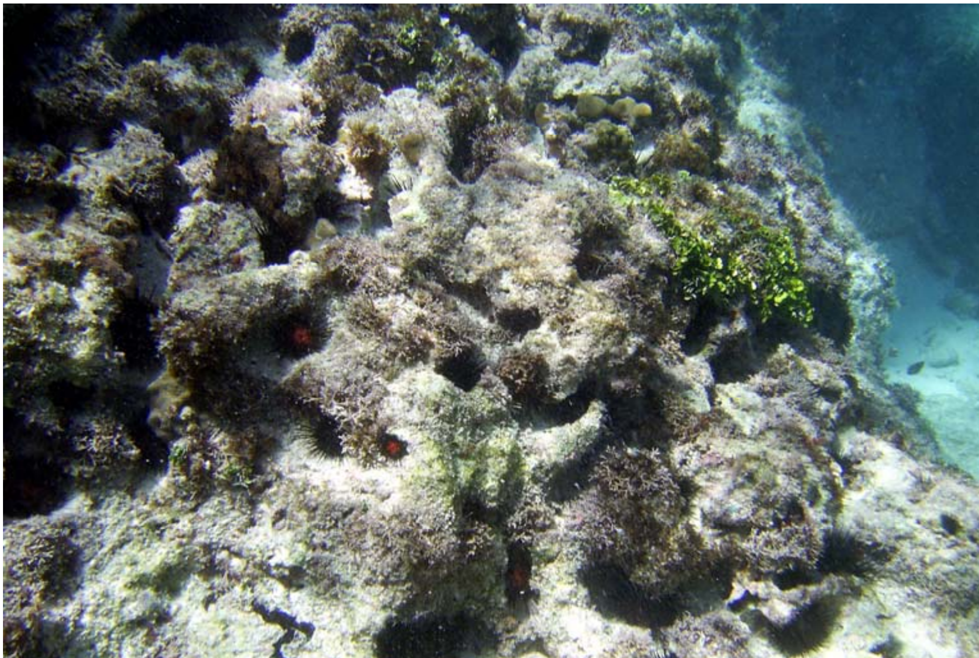

Figure 4-4. Burrows of rock-boring sea urchin (*Echinometra* sp.) pocket the subtidal surface of the ironshore .

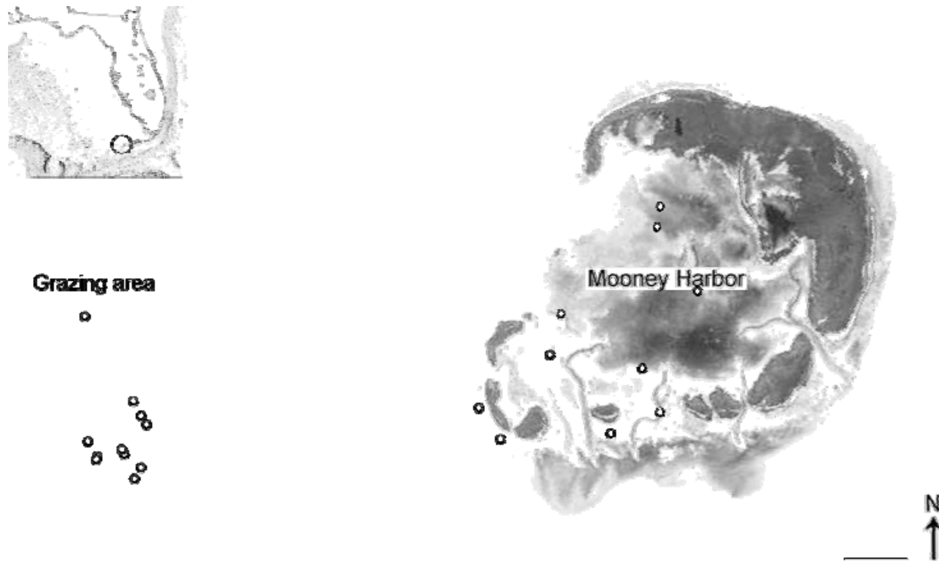

Figure 4-5. The Marquesas Keys and surrounding region. Global positional system waypoints of areas from which gastropods were collected included a large grazing area west of the islands, a small near-shore site on the outer shore of the southwestern-most islands, and Mooney Harbor lagoon. The scale bar equals 1 kilometer.

Figure 4-6. Grazing plot created by foraging *C. mydas* in seagrass beds west of the Marquesas Keys. The *Thalassia* and *Syringodium* have been cropped by grazing turtles.

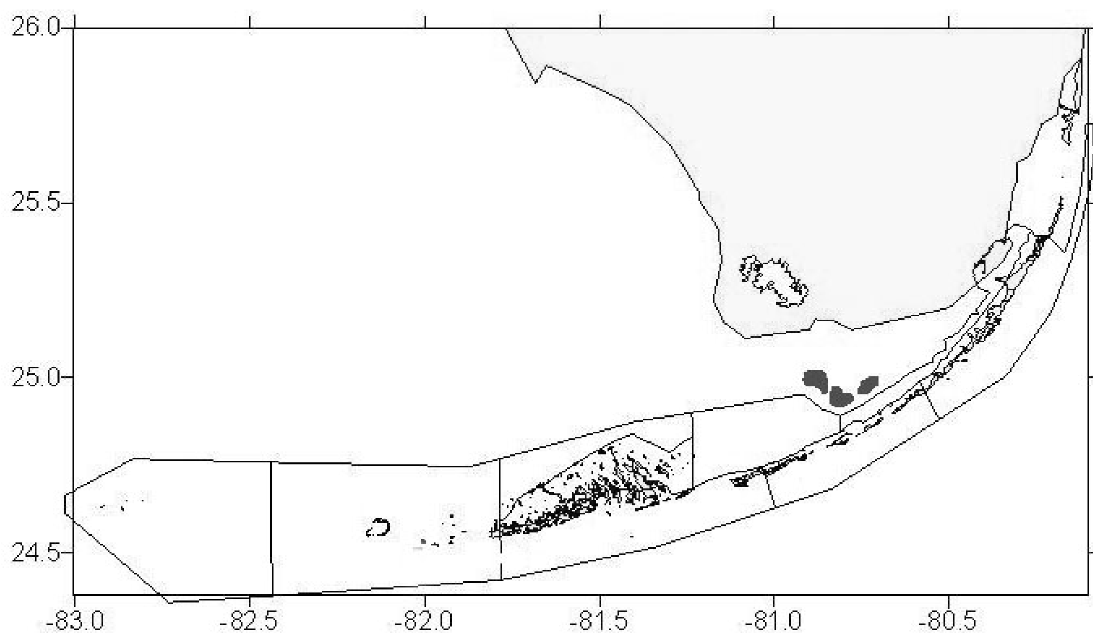

Figure 4-7. The collection areas within Florida Bay are shown here as three shaded areas, which include Twin Key Basin, Rabbit Key Basin, and Arsniker Basin.

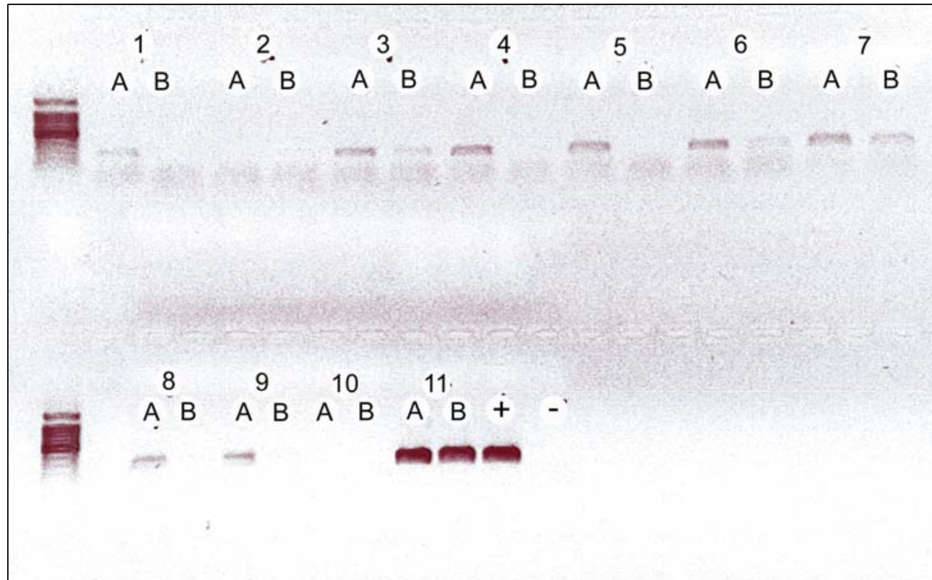

Figure 4-8. Gel electrophoreses of products produced by PCR amplification of the partial ITS2 of *Neospirorchis* species using specific primers. Each numbered sample lane pair represents 1.5 grams of *Pomacea bridgesii* tissue spiked with the following egg equivalents of *Neospirorchis* DNA: samples 1 and 2 (1 egg); samples 3, 4, and 5 (3 eggs); samples 6 and 7 (5 eggs), samples 8 and 9 (10 eggs). Sample 10 only contains snail tissue and sample 11 was extracted with a complete adult *Neospirorchis* species. The positive control (+) is *Neospirorchis* DNA and the negative control (-) is PCR reagents only. Lanes labeled A have been processed using a QIAquick spin column, whereas lanes B are have been simply diluted in TE buffer to equivalent template concentrations. The ITS2 is detected in one of the samples spiked with a single egg equivalent and all of the samples containing 3 or more egg equivalents. Brighter amplicons are observed in samples processed using the QIA quick column as compared the TE dilutions.

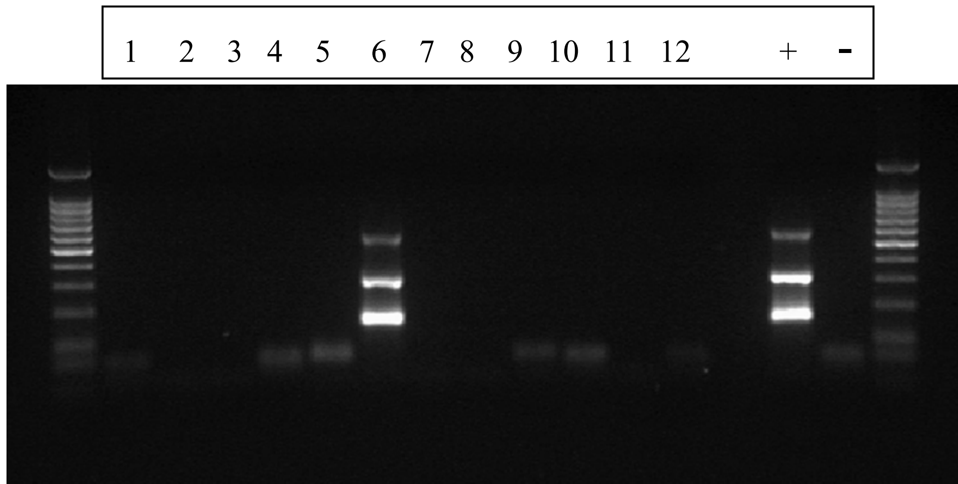

Figure 4-9. Gel electrophoresis of products obtained from PCR amplification of the trematode ITS2 from DNA extracted from the limpet *Fissurella nodosa*. The brightest band in the ladder lanes is 500 base pairs. The bright bands in lane six reflect the detection of the 300 base pair complete ITS2 using consensus primers and the smaller 175 base pair indicates specific amplification of the 5’ region of the ITS2. Products of similar size are present in the positive control lane (+), which is *Hapalotrema mistroides* DNA. Direct sequencing of the products excised from lane six confirmed the sequence to be that of Learedius learedi ITS2.

Figure 4-10. Two *Fissurella nodosa* are adhered to the intertidal zone of the ironshore at the Cayman Turtle Farm, Limited.

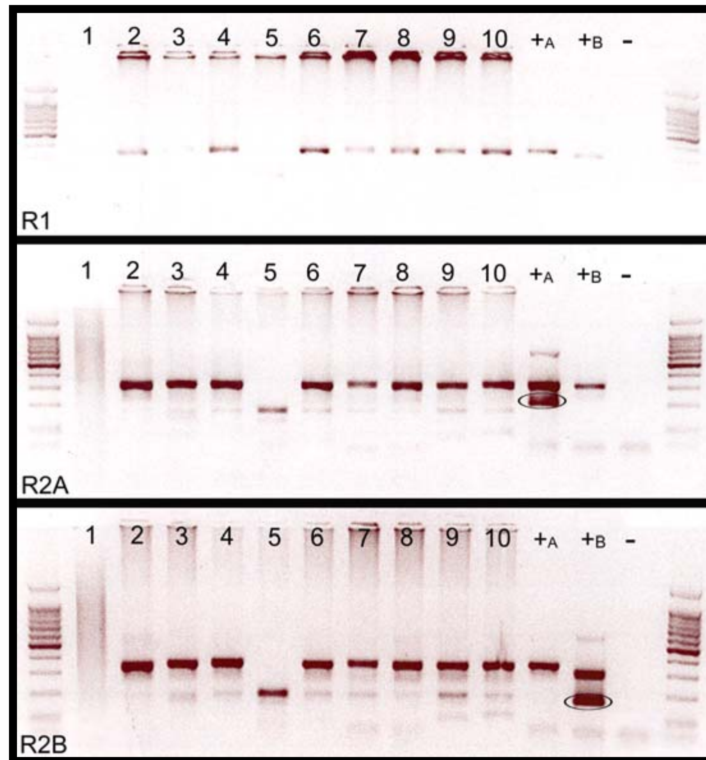

Figure 4-11. Gel electrophoresis of products resulting from hemi-nested PCR amplification of the trematode ITS2 region from the gastropod *Modulus modulus*. The R1 gel reflects amplification of trematode ITS2 using consensus primers in the first reaction. The two hemi-nested reactions R2A and R2B use spirorchiid-specific reverse primers and the bright smaller bands (black circles) in the positive controls reflect specific amplification of the 5' region of the *Neospirorchis* ITS2 (R2A) and *Hapalotrema/Learedius* ITS2 (R2B). The positive controls are *Neospirorchis* sp. (+A) and *Hapalotrema mistroides* (+B). Note that the specific primers do not amplify the non-target control.

Table 4-1. Gastropod DNA extraction protocol.

Gastropod preparation and tissue lysis
1. Remove the hepatopancreas (digestive gland) and gonad
2. Pool these tissues to a combined wet weight of 1.5 grams
3. Place wet tissue into an aluminum foil pouch and freeze in liquid nitrogen for 20 seconds
4. Crush tissues and place in 10 mls of CTAB lysis buffer with 75 µl of proteinase K (Qiagen)
5. Incubate overnight at 55º C
DNA extraction
1. Add 12 mls of chloroform:isamyl alcohol (24:1) and mix by gentle inversion
2. Centrifuge at 7,700 *G* for 30 minutes
3. Transfer aqueous phase to clean tube and repeat chloroform extraction
Precipitation
1. Add Na acetate (pH 5.4) to aqueous phase to obtain a final concentration of 0.3M
2. Add 2 volumes of 100% ethanol and mix by swirling
3. Centrifuge at 17,000 *G* for 1 hour
4. Discard supernatant and wash with 70% ethanol
5. Centrifuge at 17,000 *G* for 30 minutes
6. Dry pellet and resuspend in 1 ml of TE (pH 8.0)
DNA clean-up (pigment removal)
1. Add aliquot of DNA to QIAquick spin column according to manufacturer protocol
2. Dilute eluted DNA in TE for PCR

Table 4-2. Recipe for CTAB tissue lysis buffera.

Recipe for a 0.5 liter volume	
2% w/v CTAB	10.0 g
1.4M NaCl	41.0 g
20mM EDTA	2.9 g
100mM TrisCl	7.9 g
Fill to a volume of 0.5 L and buffer to pH 8.0	

[a]Modified from Winnepenninckx et al, 1993.

Table 4-3. PCR reaction conditions for trematode detection primers.

Parameter	Consensus trematode PCR	Spirorchiid specific PCR
Primers	SPIR1 & SPIR2	SPIR1 & NSC2 / SPIR1 & HLC4
Denaturation	95º C – 5 min	95º C – 5 min
45 cycles of:		
Denaturation	95º C – 60 sec	95º C – 60 sec
Annealing	50º C – 45 sec	56º C – 45 sec
Extension	72 º C – 120 sec	72 º C – 120 sec
Final extension	72 º C – 10 min	72 º C – 10 min

Table 4-4. Marine gastropods collected and screened for spirorchiid trematode infection from The Turtle Hospital, Marathon, Florida and surrounding area.

Species	Number
Lithopoma americanum	455
Collumbella mercatoria	596
Fasciolaria tulipa	2
Ceritheum algicola/eburneum	114
Ceritheum muscarum	115
Cerithium littoratum	8
Modulus modulus	335
Tegula fasciata	204
Turbo castanea	154
Astraea phoebia	48
Crepidula sp.	69
Total	2,100

Table 4-5. Marine gastropods collected and screened for spirorchiid trematode infection from the Cayman Turtle Farm, Limited, Grand Cayman Island, British West Indies.

Species	Number
Leucozonia nassa	292
Columbella mercatoria	310
Hemitoma octoradiata	38
Lithopoma caelatum	56
Tegula lividomaculata	11
Tegula fasciata	16
Coralliophila abreviata	10
Thais deltoidea	66
Pupurita pupa	276
Nodolittorina ziczac	360
Cenchritas muricatus	203
Nodolittorina angustior	247
Nodolittorina dilatata	273
Engina turbinella	115
Nodolittorina mespillum	317
Cerithiopsis sp.	146
Cerithium littoratum	75
Cerithium eburneum	22
Nerita versicolor	81
Nerita peloranta	46
Fissurella nodosa	550
Fissurella fascicularis	106
Fissurella barbadensis	40
Diodora listeri	43
Limpet sp.	184
Total	3,883

Table 4-6. Digenean genera detected by fecal floatation and sedimentation for samples collected from wild *C. mydas* in the Marquesas Keys region.

Sample number	Results
8/2007	
8.07.1	No parasites observed
8.07.2	*Rhytidodes* sp., unidentified immature fluke
8.07.3	*Rhytidodes* sp., presumptive *Deuterobaris* sp., *Neospirorchis* sp.
8.07.4	*Learedius* sp., *Rhytidodes* sp., unidentified immature fluke
12/2007	
12.07.1	*Rhytidodes* sp., *Rhytidodes*-like sp.
12.07.2	Unidentified trematode eggs
12.07.3	*Rhytidodes* sp., *Rhytidodes*-like sp., *Learedius* sp.
12.07.4	*Rhytidodes* sp.
12.07.5	*Rhytidodes* sp.
12.07.6	No parasites observed
12.07.7	*Rhytidodes*-like sp., *Learedius* sp.
12.07.8	*Learedius* sp.
12.07.9	*Rhytidodes*-like sp.
12.07.10	Unidentified trematode eggs
12.07.11	*Rhytidodes* sp.
12.07.12	*Rhytidodes* sp.
12.07.13	*Rhytidodes* sp.
12.07.14	*Rhytidodes* sp.
12.07.15	Presumptive *Rhytidodes* sp.
12.07.16	*Rhytidodes* sp.
12.07.17	*Rhytidodes* sp.
12.07.18	Presumptive *Rhytidodes* sp.
12.07.19	No parasites observed
12.07.20	*Learedius* sp., *Rhytidodes* sp.
3/2008	
3.08.01	*Polyangium* sp.
3.08.02	*Rhytidodes* sp., *Polyangium* sp.
3.08.03	*Polyangium* sp., *Rhytidodes* sp.
3.08.04	*Rhytidodes* sp, *Polyangium* sp. and *Schizamphistomoides* sp.
3.08.05	*Rhytidodes* sp., *Polyangium* sp.
3.08.06	*Rhytidodes* sp., *Polyangium* sp.
3.08.07	*Deuterobaris* sp., *Polyangium* sp.
3.08.08	*Schizamphistomoides* sp.
3.08.09	*Polyangium* sp.
3.08.10	*Deuterobaris* sp., *Polyangium* sp, and *Schizamphistomoides* sp.
3.08.11	*Deuterobaris* sp., *Polyangium* sp.
3.08.12	*Polyangium* sp.
3.08.13	*Polyangium* sp., *Schizamphistomoides* sp.
3.08.14	*Polyangium* sp., *Deuterobaris* sp., *Schizamphistomoides* sp.
6/2007	
6.07.01	*Polyangium* sp., *Schizamphistomoides* sp.
6.07.02	No parasites observed
6.07.03	*Learedius* sp., *Polyangium* sp.
6.07.04	No parasites observed
6.07.05	*Polyangium* sp., *Rhytidodes* sp.
6.07.06	*Polyangium* sp.
6.07.07	*Polyangium*, *Schizamphistomoides* sp.

Table 4-7. Gastropods collected from the Marquesas Keys region and Florida Bay examined by microdissection.

Specie*s*	Number examined
Marquesas Keys region	
Astraea pheobia	13
Tegula fasciata	39
Lithopoma americanum	53
Collumbella mercatoria	25
Modulus modulus	16
Cerithium atratum	69
Crepidula sp.	9
*Fasciolaria tulipa**	1
*Horse conch**	3
Cerithium littoratum	8
Turbo castanea	50
Conus sp.*	3
Patelloida pustulata	4
*Emerald nerite**	2
Total	295
Florida Bay	
Modulus modulus	92
Turbo castanea	48
Cerithium sp.	21
Lithopoma americanum	50
Tegula fasciata	67
Astraea pheobia	17
Total	295

Table 4-8. Marine gastropods collected and screened for spirorchiid trematode infection from Marquesas Keys region, Florida.

Species	Number
Grazing plots (west of Marquesas)	
Modulus modulus	263
Astraea phoebia	71
Cerithium atratum	33
Columbella mercatoria	74
Tegula fasciata	44
Lithopoma americanum	2
Crepidula sp.	85
Murex sp.	4
Fasciolaria tulipa	2
Pleuroploca gigantea	1
Conus sp.	1
Total	580
Mooney Harbor and near shore zones	
Modulus modulus	3573
Cerithium atratum	916
Lithopoma americanum	501
Turbo castanea	396
Columbella rusticoides	146
Tegula fasciata	59
Cerithium sp.	50
Crepidula sp.	38
Cerithium littoratum	27
Astraea phoebia	10
Total	5,716

Table 4-9. Marine gastropods collected and screened for spirorchiid trematode infection from Florida Bay (Twin Key Basin, Rabbit Key Basin), Everglades National Park.

Species	Number
Modulus modulus	478
Cerithium eburneum	270
Cerithium sp.	20
Turbo castanea	32
Columbella rusticoides	58
Zafrona taylorae	105
Tegula fasciata	50
Lithopoma americanum	450
Total	1,463

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BIOGRAPHICAL SKETCH

Brian Stacy was born in Stone Mountain, Georgia in 1975. He developed a love of the outdoors and wildlife at an early age growing up in north Georgia and periodically spending time at the family farm in rural Montgomery county. A special interest in reptiles developed early on. He attended undergraduate school at the University of Georgia during which time he worked as a farm hand at the University dairy and on a private farm working with a large collection of psittacines. He completed an externship with the Puerto Rican Parrot Project under the Department of Natural Resources.

After deciding to pursue a career in wildlife health and conservation, he was admitted into veterinary school at UGA where he received his Doctor of Veterinary Medicine degree in 2001. Dr. Stacy worked with captive and free-ranging wildlife throughout veterinary school and completed two projects abroad at the Madras Crocodile Bank Trust, Institute for Herpetology. He also worked as research assistant with the Southeastern Wildlife Disease Study and completed externships with the Armed Forces Institute of Pathology, Wildlife Conservation Society, and Western College of Veterinary Medicine, Saskatoon. Following veterinary school, Dr. Stacy completed a residency program in anatomic pathology at the University of California at Davis, Veterinary Medical Teaching Hospital and the Zoological Society of San Diego, and became a board certified pathologist in 2004. His interest in health and disease in free-ranging wildlife led him to pursue the study of marine spirorchiids and their effects on sea turtles.

Currently, Dr. Stacy is a veterinary pathologist with the UF Aquatic Animal Health Program. He studies and works as a diagnostician and consultant on health and disease related issues affecting sea turtles, marine mammals, and other aquatic species. He also is involved in a number of other wildlife health projects within the US and abroad.