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Cryptic Speciation Among Meiofaunal Flatworms

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CRYPTIC SPECIATION AMONG MEIOFAUNAL FLATWORMS

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By

Henry Joseph Horacek

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To the Dean of the Graduate School:

We are submitting a thesis written by Henry Joseph Horacek entitled Cryptic Speciation among Meiofaunal Flatworms.

We recommend acceptance in partial fulfillment of the requirements for the degree of Master of Science in Biology

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Abstract:

Meiofauna are unusual in that they typically have widespread distribution, despite being small-bodied and unable to swim. This is known as the meiofaunal paradox; cryptic speciation has been suggested as a possible explanation for the meiofaunal paradox. In order to determine whether cryptic speciation and genetic differentiation across populations of meiofaunal flatworms could be detected along the eastern coast of North America, I collected specimens of two different morphospecies of meiofaunal flatworms (*Paramonotus sp.* and *Proschizorhynchella sp.*) from different locations in North Carolina and Florida. Specimens were sequenced for four different genetic markers: 18S gene, 28S gene, ITS region, and cox-1 gene. For *Proschizorhynchella sp.* I found evidence that *Proschizorhynchella sp.* consisted of two cryptic species: one in Onslow Bay, NC and one in Florida. For *Paramonotus sp.* I found that all populations consisted of the same biological species (all populations of *Paramonotus sp.* were found in Onslow Bay). I also found that 18S and 28S showed no variation across populations for both morphospecies and that the ITS region showed no genetic variation across populations for *Paramonotus sp.* There was, however, variation across the different populations of *Proschizorhynchella sp.* for both the ITS region and the cox-1 gene. For *Paramonotus sp*. there was some genetic variation across populations for the cox-1 gene. For the two sites where both morphospecies were present, there was a numerical difference in the amount of genetic variation across these two sites for these two morphospecies, wherein the variation across sites was numerically higher for *Proschizorhynchella sp.* than it was for *Paramonotus sp*.

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Introduction:

The "species" is the most fundamental taxonomic category to which a living individual is assigned in biology (Mayr 1982). Since the study of biology necessitates a method for sorting individuals into a hierarchical system of classification (Dobzhansky 1935), the delimitation of species is essential to all aspects of biology. Cryptic speciation, however, can complicate species delimitation and can lead to an underestimation of the true biodiversity of taxa (Salgado-Salazar et al. 2013, Leasi et al. 2016). Cryptic species are two or more distinct species that have been erroneously classified under one species name because they are morphologically indistinguishable from one another (Bickford et al. 2007, Saez et al. 2003). Cryptic speciation has been observed in many groups of organisms, including fungi (Salgado-Salazar et al. 2013, Crespo and Lumbsch 2010), frogs (Stuart et al. 2006), crustaceans (Belyaeva and Taylor 2009), corals (Ohki et al. 2015), and meiofauna (Scarpa et al. 2016, Jorger et al. 2012, Van Steenkiste et al. 2018).

Meiofauna are organisms that are small enough to live between sand grains, and they inhabit both freshwater and saltwater. They are considered to be larger than microfauna yet smaller than microfauna, and, generally, their size range is between a millimeter and 44 micrometers. Meiofauna (or meiobenthos) is recognized as its own ecological group and does not belong to any specific taxon. Rather, any species within a certain size range is considered to be meiofauna (Giere 2009).

Currently, it is recognized that 29 out of 35 phyla of metazoans contain meiofaunal species (Fonseca et al. 2017). This means that over two-thirds of metazoan phyla contain meiofaunal species, and there are some phyla that are exclusively meiofaunal, such as Priapulida and Gastrotricha (Giere 2009). Although the term "fauna" seems to imply that only metazoans can be meiofauna, there are several groups of protists that are considered to be meiofauna. These include Foraminifera, Heliozoa (or Actinopodia) (now mostly a descriptive term), Amoebozoa, Ciliata, and microalgae (e.g. diatoms) (Giere 2009).

Marine meiofauna are an important component of marine environments because of their high levels of species richness and biodiversity (Kennedy and Jacoby 1999). Moreover, they are abundant in a variety of different marine environments, including the abyssal trenches and plains (Tselepides and Lampadariou 2004), the muddy intertidal environment (Heip et al 1985), and sandy beaches (Mantha et al. 2012). Meiofauna also contribute to ecosystem functions and ecosystem services (Schratzberger and Ingels 2017). Ecosystem functions and services pertaining to meiofauna are important due to the fact that the marine benthic environment is the largest habitat on Earth in terms of surface area (Snelgrove 1997). For instance, bioturbation by meiofauna increases denitrification of sediment, which is an important part of the nitrogen cycle (Bonaglia et al 2014). Thus, these benthic creatures can be considered to be economically as well as ecologically important (Schratzberger and Ingels 2017).

Meiofauna have an unusual property in that many species have widespread geographic ranges, despite being small-bodied and unable to swim. Many benthic

animals, such as macrofaunal echinoderms (e.g. sea stars), have a pelagic larval stage. This allows larvae to swim into the water column and disperse, even though the adults are still benthic and have limited mobility. Meiofauna, perhaps due to their size, have no pelagic larval stages and thus have no obvious means of dispersal (Giere 2009). One would assume that this isolation would cause evolutionary divergence due to a lack of interbreeding between populations. Even in the absence of geographic barriers, a large enough geographic distance should theoretically create enough reproductive isolation for the occurrence of speciation, provided that populations are discontinuous. This does not appear to happen with the meiofauna because many meiofaunal species have wide-ranged or cosmopolitan distributions. This phenomenon is what is known as the "meiofaunal paradox," (Boeckner et al. 2009, Giere 2009, Artois et al. 2011).

There have been several explanations for the meiofaunal paradox. One idea is that meiofauna are able to spread far and wide because they become suspended in the water column and passively get swept from place to place. Several species of meiofauna have been observed suspended in the water column, and it is assumed that most species of meiofauna are swept into the water column due to bioturbation or water currents (Palmer 1988, Boeckner et al. 2009, Thomas and Lana 2011). Boeckner et al. (2009) also provided evidence that several species of meiofauna are suspended in the water column and that this may allow them to travel long distances. Hagerman and Rieger (1981) found evidence that meiofauna are frequently suspended in the water column.

Another explanation is the "everything is everywhere," hypothesis or "ubiquity" hypothesis. The maxim of this hypothesis is that "everything is everywhere, but the

environment selects." This is the hypothesis that microscopic organisms are widely dispersed because they are able to travel passively, as opposed to larger organisms that are supposedly hampered by their size and restricted to certain geographic areas. This hypothesis was later cited as a possible explanation for the meiofaunal paradox (Fontaneto and Hortal 2013, Artois et al. 2011, Meyer-Wachsmuth et al. 2014). However, when isolated populations have been studied at the genetic level, it is often found that widely-separated members of putative species are genetically different (Casu and Curini-Galletti 2006, Leasi and Norenburg 2014, Cabezas et al. 2013, Jorger et al. 2012, Salgado-Salazar et al. 2013, Scarpa et al. 2016, Leasi et al. 2016).

Meiofauna do have some means of dispersal, despite the lack of a pelagic larval stage. There are four methods: erosion of sediment, active emergence from the sediment, rafting on the surface of the water, and re-entry. Erosion is simply the mechanism wherein meiofauna are washed away after the sediment has been disturbed. Emergence is when the animals actively crawl out of the sediment and allow themselves to be washed away by the flow of water (Giere 2009, Thomas and Lana 2011). Once suspended in the water column, marine meiofauna can be swept into water currents and passively travel long distances (Boeckner et al. 2009, Thomas and Lana 2011).

The next step after emergence is "rafting." Sometimes, bacteria and microalgae on the ocean floor can excrete mucous that aggregate and form dense mats, which can become suspended, forming "rafts." This rafting can be used as a means of dispersal among certain meiofauna, such as copepods, diatoms, ciliates, and nematodes (Giere 2009). Another form of transportation is "re-entry." This is where suspended meiofauna

settle back into the sediment. It was once thought to be passive and random, but recent studies have shown that some meiofauna are capable of selective re-entry (Giere 2009). Mevencamp et al. (2016) found that some groups of meiofauna (nematodes, copepods, and nauplius larvae) are capable of influencing the direction of re-entry and were even able to select the substrate that they would land on.

Although there are several groups of marine meiofauna, I have chosen to study meiofaunal flatworms for this project. Meiofaunal flatworms are free-living platyhelminths, which are commonly known as turbellarians. The term "Turbellaria" is now recognized as a paraphyletic group and is no longer a valid taxon. However, it is often used as a descriptive term when discussing free-living flatworms. Turbellarians are also among the most primitive of metazoans, and there are several more species left to be identified and described (Giere 2009). In fact, Appeltans et al. (2012) estimated that there could be over 29,000 unknown turbellarian species.

For marine meiofaunal flatworms, it is thought that the main mode of transportation is “emergence,” and they are often observed suspended in the water column (Palmer 1988, Boeckner et al. 2009). Some flatworms are known to travel by “raft” (Goldstein et al. 2014), but I could not find any references to any species of meiofaunal flatworms that have been observed on these “rafts.”

This poses four major questions. First, can one detect cryptic species across geographically distant populations of putative species? Second, is there a genetic gradient across different populations of the same species of meiofaunal flatworm? Third,

will different morphospecies show different genetic divergence across populations? Fourth, will different genetic markers show different variations across populations of morphospecies of meiofaunal flatworms?

Because I am looking for cryptic species, I cannot rely upon morphological characteristics for species delimitation, although I will be using them to identify putative species. Cross-breeding experiments have been done successfully in a study by Scarpa et al. (2016), but that study was done in conjunction with several other methods of species delimitation, including molecular species delimitation. This would be a very large study and would honestly constitute its own thesis project. In addition, I would argue that cross-breeding experiments are not sufficient by themselves because they are done in artificial settings. Also, sometimes separate species can interbreed. Therefore, molecular species delimitation is the most appropriate method for this study.

Biologists can determine whether two or more individuals belong to the same biological species by looking at specific regions of DNA, called genetic markers. Usually, scientists use mitochondrial DNA to identify cryptic species, but ribosomal markers are typically used for flatworms. This is because the most commonly used mitochondrial genetic marker, the mitochondrial gene Cytochrome c Oxidase subunit I (cox-1), is so variable in flatworms that the primers often have to be taxon-specific (Van Hove et al. 2013). However, there is also evidence that ribosomal markers might underestimate meiofaunal diversity (Tang et al. 2012), so using only rDNA genetic markers may be unwise. Using a combination of morphological taxonomy and molecular species delimitation tools, Tang et al. (2012) found evidence that environmental DNA

(eDNA) surveys that use the 18S marker may be underestimating meiofaunal diversity, and they suggested that the cox-1 marker may be more appropriate. Note that Tang et al. (2012) did not do any morphological taxonomy and that all of the data were mined from GenBank. Thus, the quality of these sequences may vary across different authors and different studies.

In this study, I used the cox-1 gene in addition to three ribosomal markers (18S, 28S, ITS region). I used a single-gene method for delimitation, as opposed to a multi-gene method. In other words, each genetic marker was tested separately for the presence of cryptic species. A multi-gene delimitation method requires the incorporation of multiple genetic markers into one test (Van Steenkiste et al. 2018). I did not use a multi-gene approach because, in my opinion, all specimens would have to have all four genetic markers sequenced. I think this because individuals must be grouped into hypothetical species that were estimated based on the single-gene method, and there is no guarantee that each individual in the population has identical haplotypes for each marker. Either due to food in the intestine of the sample or due to insufficient concentration in the PCR product, I was not able to have all four markers for every specimen. Instead, I elected to obtain three sequences of each genetic marker for each population, although they are not necessarily from the same specimens.

In recent years, there has been a rise in interest of using cryptic speciation to explain the meiofaunal paradox (Casu and Curini-Galletti 2006; Leasi and Norenburg 2014; Scarpa et al. 2016; Jorger et al. 2012). However, few papers have compared the efficacy of using mitochondrial DNA (mtDNA) markers against ribosomal DNA (rDNA)

markers when assessing meiofaunal flatworm diversity, with the exception of Tang et al. (2012), Leasi et al. (2016), and Van Steenkiste et al. 2018. Also, Tang et al. (2012) was looking at eDNA and was not investigating cryptic speciation. Instead, they were assessing the validity of markers by comparing them to the morphology of putative species, which may not be the best method if cryptic speciation has occurred, since cryptic species have no morphological differences. Leasi et al. (2016) conducted a similar study, but they were only looking at one genus of meiofauna, as opposed to my thesis which includes two morphospecies from different orders. Van Steenkiste et al. (2018) used both single-gene and multi-gene delimitation methods to compare 18S, 28S, and cox-1 for a single putative species. While their multi-gene method may provide a more high-definition comparison, they did not look at the ITS region, and they did not look at two different putative species. My study provides valuable insight by comparing two putative species from two separate orders. Additionally, Carbayo et al (2017) looked at the land planarian but only looked at ITS-1 and cox-1; also, it is not a meiofaunal species.

There are a few different methods for molecular species delimitation using a single genetic marker. First, there is DNA taxonomy through DNA barcoding, which involves using a predetermined nucleotide threshold above which individuals are defined as being heterospecifics and below which they are defined as being conspecific (Fontaneto et al. 2015). The problem with this method is that it is subjective and does not take into account the particular taxon that is being examined. Second is K/θ, which assumes that sister clades of a particular genetic marker have a 95% chance of belonging

to different species if the interclade divergence (K) is at minimum four times greater than the intraclade variation (θ). This method does not use sequence data as an input, rather the user must create distance matrices and identify sister clades. Third, there are the delimitation methods based on the generalized yule-coalescent model (GYCM). These methods require the construction of ultrametric trees. Next, there is the Poisson tree process model, which also requires the construction of a tree (Fontaneto et al. 2015). Due to my lack of experience creating these trees, it seemed to me that these were not the best options. Then, there are haplowebs, which relies on mutual allelic exclusivity, so it does not work for mtDNA and rDNA. For instance, cox-1 and 18S genes appear to only have one allele. Lastly is Automatic Barcode Gap Discovery (ABGD), which is a method of molecular species delimitation that uses DNA barcoding. ABGD attempts to determine the best threshold for a given set of individuals. If an adequate threshold cannot be detected, then the individuals are considered to belong to the same species (Fontaneto et al. 2015). In other words, ABGD is used to calculate automatically species hypotheses without *a priori* knowledge (Puillandre et al. 2012). Also, it only requires sequence data to run this statistical test, so it is very good for the inexperienced molecular biologist. I used the ABGD website (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to run these tests.

ABGD works by automatically sorting sequences into hypothetical species based on the barcode gap, which is detected when the divergence among conspecifics is significantly smaller than the divergence among heterospecifics. Essentially, ABGD creates a nucleotide distance threshold below which individuals are considered

conspecifics and above which they are considered to be heterospecifics (Puillandre et al. 2012). Since ABGD can take sequence alignment as input (Puillandre et al. 2012), it is well suited for my study. It cannot perform multi-gene delimitation, but, as I explained earlier, I do not have a sufficient number of samples to do that.

One advantage of ABGD is that it does not rely upon a single speciation model but takes into account four different speciation models: a radiation model, a Moran model, a Yule model, and a critical model (Puillandre et al. 2012). The radiation model is characterized by a speciation even that occurred once where all species radiated. In the Moran model the number of species is constant because an extinction event is followed by the creation of a new species. Both the Yule model and the critical model are branching, so that the speciation rate and the extinction rate are different. The difference between the Yule model and the critical model is that in the Yule model the extinction rate is at zero (no extinction), but in the critical model the extinction rate is the same as the speciation rate, so that the number of species in a group remains the same. This puts it at an advantage over Generalized Mixed Yule-Coalescence Model (GMYC), which relies heavily upon the Yule model of speciation (Fontaneto et al. 2015). Also, since it is not a tree-based method, it does not require monophyly, as GMYC does (Fontaneto et al. 2015). This is important because there is evidence that 15-40% of groups of animals are not monophyletic in mitochondrial gene trees (Fontaneto et al. 2015). ABGD has been previously used to delimit meiofaunal species (Jorger et al. 2012, Tang et al. 2012, VanSteenkiste et al. 2018).

In order to answer my second question (is there a genetic gradient?), I plan to use Analysis of Molecular Variance (AMOVA) to measure gene flow, since it can be used to compare intrapopulation genetic diversity to interpopulation genetic diversity within species (Excoffier et al. 1992). The hierarchical method works by looking at diversity across regions, diversity across populations within regions, and diversity within populations. A non-hierarchical approach can also be used to look at variance between populations and within populations (Excoffier et al. 1992). In other words, one places all populations into one group. I used a non-hierarchical approach, since my study is too small to use a hierarchical approach (Fitzpatrick 2009).

As to my third question (will different morphospecies show different genetic divergence across populations?), the best way to do this is to compare AMOVA results between the two morphospecies for each genetic marker to see if there is a numerical difference in the variation across populations. There is no statistical test that I am aware of that allows you to compare AMOVA results, so I decided to see if there was a numerical difference between them.

For my fourth question (will different subunits of ribosomal DNA [18S, 28S, ITS-1/5.8S/ITS-2] and mitochondrial DNA [cox-1] show different variations across populations of morphospecies of meiofaunal flatworms?), the best way, in my opinion, would be to look at the results of ABGD and AMOVA for each genetic marker for each specimen and look to see if there is a numerical difference in genetic variation for each genetic marker. The idea here was to see if there was one specific marker that could show the most genetic variation for use in future studies. Originally, I had hypothesized

that mtDNA would be a better indicator of diversity for flatworms than ITS region. However, I soon realized that since I am working with cryptic species, it would be difficult to assess. Perhaps with a larger sample size, I could determine whether one marker is better than the other. After all, it is difficult to determine whether one is underestimating diversity or overestimating it without morphological data or a larger sample size. Van Steenkiste et al. (2018) showed a good way of determining the fidelity of certain genetic markers, but that study had a much larger sample size than I was able to obtain. That study also had clear morphological differences in the pseudo-cryptic species.

The primary goal of my study is to obtain and compare ribosomal DNA sequences (18S, 28S, and ITS-1/5.8S/ITS-2) and mitochondrial DNA sequences (Cytochrome c Oxidase subunit 1 [cox-1]) for three representatives of each of my target species at spatially separated sites. I am looking at different markers because there is evidence that one marker is not adequate for species delimitation (Dupuis et al. 2012). In addition, I analyzed the genetic similarity within each population and across populations for genetic divergence. In doing this, I hope to gain more insight into the evolution of marine meiofaunal flatworms and possibly identify any cryptic species in the region.

My target species are two undescribed species of marine meiofaunal flatworms found along the east coast of North America: *Paramonotus sp*. and *Proschizorhynchella sp*. Although these species are officially undescribed taxonomically, they are still good candidates for this study for several reasons. Through unpublished data and personal communication with Dr. Julian Smith, I know the basic morphology of both

morphospecies. These two morphospecies are appropriate for a comparison because they are morphologically distinct from each other because they belong to different orders. *Paramonotus sp.* belongs to Proseriata (Family: Monocelididae), and *Proschizorhynchella sp.* belongs to Rhabdocoela (Suborder: Kalyptorhynchia). Thus, it is relatively easy to identify these putative species with a stereomicroscope. Additionally, *Paramonotus sp*. is thought to be a mobile species of flatworm that allows itself to be swept to different locations by the tide, and *Proschizorhynchella sp.* is thought to be a relatively stationary species that resists being swept away by the tide (personal communication, Julian Smith). This is because *Proschizorhynchella sp*. has three adhesive belts that it uses to grab onto sand grains and make a "weight-belt." *Paramonotus sp.* appears to be confined to Onslow Bay in North Carolina, but *Proschizorhynchella sp.* is widespread, having populations extending from Cold Spring Harbor, New York to Northern Florida (personal communication, Julian Smith).

These questions led to the formation of different hypotheses. First, will a morphospecies of meiofaunal flatworm prove to be two or more species, provided that populations are sufficiently distant from each other? I hypothesized that populations of each putative species will prove to be two different species if my populations are sufficiently distant from each other. I reasoned that, although there are no geographic barriers between my populations, a sufficient amount of geographic distance should lead to reproductive isolation because meiofauna lack any obvious means of dispersal. If there were no cryptic species to be found, then I reasoned that there must not be a large enough distance between populations.

Second, will there be a genetic gradient across populations of a morphospecies of meiofaunal flatworm that are geographically distant from one another? I hypothesized that genetic variation in the 18S gene (rDNA), 28S gene (rDNA), ITS region (rDNA), and the cox-1 gene between populations of *Paramonotus sp.* and between populations of *Proschizorhynchella sp.* will be significantly different from the intrapopulation genetic variation, even if cryptic speciation is not observed. In other words, AMOVA should show that there are significant genetic differences across these populations along a spatial gradient. I reasoned that gene flow should decrease as geographic isolation or distance increases. If the genetic differences are not significant according to AMOVA, then we can assume that genetic differentiation across populations is not significant. Now, this does not exactly test for a genetic gradient *per se*, but if there is no genetic differentiation then, in my opinion, there is no genetic gradient. If there is genetic gradient, we could then look for a numerical trend to see if there is a genetic gradient.

Third, will different subunits of ribosomal DNA (18S, 28S, and ITS region) and mitochondrial DNA (cox-1) show different variation across populations of morphospecies of meiofaunal flatworms? I hypothesized that the 18S gene will show the least amount of variation across populations, the 28S gene will show slightly more variation, the internal transcribed spacer (ITS-1/5.8S/ITS-2) will show even more variation, and the cox-1 gene will show the most variation across populations. The cox-1 gene (mtDNA marker) should show more variability than rDNA markers because there is some evidence that ribosomal DNA underestimates the biodiversity of meiofauna (Tang et al. 2012). I also thought the 18S gene will be less divergent than 28S because the 28S

gene in meiofaunal flatworms has a higher mutation rate than 18S (Scarpa et al. 2015). I thought that the ITS region should show the most variation of the ribosomal DNA because the internal transcribed spacers (ITS-1 and ITS-2) do not code for ribosomal DNA.

Fourth, will different morphospecies show different genetic divergence across populations? I hypothesized that *Proschizorhynchella sp.* would have a significantly greater genetic variation across populations than *Paramonotus sp.* because one is thought to be a relatively stationary species while the other is thought to be a relatively mobile species. Theoretically, a mobile species should show less genetic variation than a stationary species, since higher mobility leads to higher gene flow between populations. I planned to address this question by running AMOVAs on the sites where both morphospecies were present and seeing if there was a numerical difference in the variation across populations.

Materials and Methods:

Collection Sites:

Specimens were collected from six different sites, the majority of which were located in Onslow Bay, North Carolina. Originally, the plan was that I would travel to Onslow Bay to collect specimens from four sites that were about 10 kilometers apart from each other. These sites included Iron Steamer Pier, Emerald Isle, North Topsail Beach, and Wrightsville Beach (Figure 1). I did not find *Proschizorhynchella sp.* at all four locations, but Dr. Julian Smith was able to collect samples from Oak Island, NC and from the coast of Florida, near the Whitney Laboratory for Marine Bioscience; these sites did have *Proschizorhynchella sp.* (Figure 1, Figure 2). All samples were collected during the summers of 2016 and 2017, with the exception of the Florida samples, which were collected in May of 2016.

Iron Steamer Pier is the location of a destroyed pier on Bogue Banks, which is an island. The GPS coordinates are 34°41’33”N 76°49’47”W. Emerald Isle is a small town on the south-western side of Bogue Banks, and samples were taken from a site that is to the southwest of the town, near the western point of the island. The GPS coordinates are 34°64’48”N 77°08’72”W. North Topsail Beach is located on its own island (GPS coordinates are 34°43’13”N 77°53’38”W), and Wrightsville Beach is also on its own island near Wilmington, NC (GPS coordinates are 34°20’97”N 77°79’13”W). Oak Island is an island in North Carolina located outside Onslow Bay, and the GPS

coordinates are 33°91’38”N 78°18’41”W (Figure 1). Specimens from Florida were collected approximately 100 meters north of Marineland Dolphin Adventure (GPS coordinates are 29°67’14”N 81°21’32”W), which is across the street from the Whitney Laboratory for Marine Bioscience (Figure 2).

For each specimen, the date of collection and the position on the beach were recorded. The “waypoint” and collection site were additionally recorded with GPS. The “waypoint” refers to the GPS location of the actual location within the collection site from which the samples were taken. Sand samples were taken from three different positions within each waypoint: the “wave-break,” the “swash,” and the “shiny.” The wave-break is the position where the waves break, the swash is where the water runs up the beach, and the shiny is where the sand still retains some water. Occasionally, we would take samples from the “bore-line” which is where the waves swell up before the break. This was not done often because of the depth of the water. The date and location of each specimen was recorded, but I did not incorporate these data into my analysis.

At least three specimens for each target species were taken from each collection site. I attempted to use the same three specimens per morphospecies for all four genetic markers, but often, either due to food in the specimen’s intestine or insufficient amplification from low template concentration, I needed to extract DNA from more than three specimens per morphospecies for each site. Thus, I had planned to have three consensus sequences for each genetic marker for each species at each collection site, but they are not necessarily from the same specimen.

Isolating Specimens from Sediment:

I isolated specimens by first putting the sand into a large Erlenmeyer flask and filling the flask with magnesium chloride ($MgCl_2$), which puts the organisms into a state of paralysis. Then, I inverted the flask a few times to shake up the sand. The $MgCl_2$ was then poured into a 63µm sieve, so that only the meiofauna were left; I could then wash them into a petri dish with filtered seawater. This technique is described by Hulings and Gray (1971). The saltwater I used was natural seawater that had been filtered at the UNC Marine Lab. Specimens were identified with a stereomicroscope. Next, each specimen was preserved in an Eppendorf tube filled with 1000µL of 100% ethanol.

Extracting DNA from Specimens and Sequencing:

I extracted DNA from specimens preserved in ethanol using the Qiagen DNeasy Tissue Kit (Appendix A). Next, I used Polymerase Chain Reaction (PCR) to amplify the desired segments of DNA. Afterwards, the PCR products were purified with a MinElute Kit (Appendix B). Then, sequences were prepped and placed into sequencing tubes with the appropriate primer, according to the instructions provided by Eurofins Genomics. Sequencing tubes were purchased from Eurofins and were shipped overnight for Sanger sequencing.

For DNA polymerase, I used either Phusion Taq 2X master mix (prepared from the "Phusion High-Fidelity PCR Kit" purchased from New England BioLabs, Inc.) or Taq 2X master mix (purchased from New England BioLabs, Inc.) for PCR. Phusion Taq was used when possible, but I did not start using it until about half way through the study,

and it is also more expensive. For the cox-1 gene, I only used Taq standard (the Taq 2X master mix) because the primers that I used (MICOIintF and jgHCO2198) were degenerate primers (Table 1), so finding the correct annealing temperature for Phusion Taq would take some trial and error because Phusion Taq requires a different annealing temperature. For the ITS sequences, I only used Taq standard because it took me a very long time to create those primers, and I just did not have the time to find the optimal annealing temperature for Phusion Taq for all of those primers. For 18S and 28S, I used both Phusion Taq and Taq standard (Appendix C).

PCR and Primers:

<u>18S gene:</u>

For Taq 2x master mix, one of two programs was used. The first was what I called "NEB18S," which has an initial denaturation at 95°C for 30 seconds and 34 cycles of the following: denaturation at 95°C for 20 seconds, annealing at 52°C for 25 seconds, and elongation at 68°C for two minutes. Then, there was a final elongation at 68°C for 5 minutes. The second thermal cycler program was "18STAQ"," which has an initial denaturation at 95°C for 30 seconds and 35 cycles of the following: denaturation at 95°C for 20 seconds, annealing at 50°C for 25 seconds, and elongation at 68°C for two minutes. Next, there was a final elongation at 68°C for 5 minutes (Appendix C).

For Phusion Taq, only one program was used. It was called "JS3TIMPF" and has an initial denaturation at 98°C for 30 seconds and 35 cycles of the following: denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and elongation at

72°C for one minute and thirty seconds. Then, there was a final elongation at 72°C for 5 minutes. (Appendix C).

For 18S, I used TimA as a forward primer and TimB as a reverse primer to create an amplicon for the internal primers: TimA, 600R, 1100F, and HNRV (Table 1). The TimA/TimB amplicon was about 1700 base pairs (Figures 3 and 4). The stock primers were mostly 20 µM, as opposed to the 10µM stock primer concentration that NEB (New England BioLabs) suggests for 25 µL PCR reactions (See https://www.neb.com/protocols/2012/09/13/protocol-for-taq-2x-master-mix-m0270). This means that the stock primers were diluted to 20 µM, not the PCR mix; I did not change the volume of the PCR reaction. However, towards the end I made 10uM primers, since I realized at some point that that was suggested (Appendix C). Next, I ran another two separate PCR reactions for the internal primers. I expected the TimA/1100R amplicon to be about 1100 bp (base pair) long, and I expected the 600F/HNRV amplicon to be about 1200 bp long. 1100F and 600R were only used for sequencing primers. The fragments were overlaid to form a two-fold consensus sequence (Figure 4). For sequencing, I used sequencing primers TimA, 600R, and 1100R for the TimA/1100R amplicon, and I used 600F, 1100F, and HNRV for the 600F/HNRV amplicon (Figure 5).

28S Gene:

For 28S, I used the same thermal cycler programs (JS3TIMPF for Phusion Taq; NEB18S and 18STAQ for Taq standard) because the primers had almost the same annealing temperature, as calculated by the NEB website

(https://tmcalculator.neb.com/#!/main). I used both Taq standard and Phusion for the DNA polymerase (Appendix C). As with 18S, I used 20uM primers at first but then switched to 10uM primers. I first ran a PCR with the LSU5 and LSUD6-3 primers (Figure 6, Table 1). This amplicon was approximately 1690 bp long. The internal primers were Poly28SF1 and Poly28SR2 (Figure 7, Table 1). The LSU5/LSUD6-3 amplicon was usually diluted 1:10, but sometimes I left it undiluted if I deemed that the concentration of the PCR product was too low. The internal amplicon was about 1200 bp long. If I made a two-fold consensus, this gave me a consensus sequence about 1200 bp long. I used the LSU5/LSUD6-3 amplicon to help with the two-fold consensus sequence in all but one case, where the LSU5/LSUD6-3 amplicon was too dilute to send out for sequencing; I did this because the other specimens (besides the ones I had already used) had double sequences from food in the specimen's intestine (Appendix C). The sequencing primers were the same as the PCR primers (Figure 8).

Cox-1:

Only standard Taq was used, and two thermal cycler programs for standard Taq were used. LERAY5 has 35 cycles of the following: denaturation at 95°C for 10 seconds, annealing at 46°C for 30 seconds, and elongation at 72°C for one minute. Then, there was a final elongation at 68°C for 5 minutes. LERAY3, which had less amplification, has 25 cycles of the following: denaturation at 95°C for 10 seconds, annealing at 46°C for 30 seconds, and elongation at 72°C for one minute (Appendix C).

The dilution of primers was 20uM for all of these. For this one, I had only one amplicon, using these primers: MICOIintF (forward primer) and jgHCO2198 (Table 1). My consensus sequence was about 225 bp long (Figure 9). This primer set lands what I shall refer to as the "Folmer region," and this region is a section of the cox-1 gene. This region is referenced as such because Folmer et al (1994) created a primer set that amplified a section of cox-1 gene; these primers are widely used for many invertebrate taxa (Leray et al. 2013). Leray et al. (2013) created a forward primer (MICOIintF) that fell within the Folmer region that could be paired with the reverse Folmer primer (jgHCO2198) to amplify a region that is about 250 bp long. This is the primer set that I used.

ITS Region:

For the ITS region, I used only Taq standard. The programs I used were ITSTAQ7 and ITSTAQ8. ITSTAQ7 was used for *Paramonotus sp.* and ITSTAQ8 was used for *Proschizorhynchella sp.* (Appendix C).

The ITSTAQ7 program had an initial denaturation at denaturation at 95°C for 30 seconds and 35 cycles of the following: denaturation at 95°C for 20 seconds, annealing at 55°C for 25 seconds, and elongation at 68°C for 3 minutes. There was a final elongation at 68°C for 5 minutes. This was for the ITSFwd/ITSLZ amplicon and ITSBJFwd/ITS2.2 amplicon for *Paramonotus sp.* (Figure 10, Table 1).

ITSTAQ8 program had an initial denaturation at denaturation at 95°C for 30 seconds and 35 cycles of the following: denaturation at 95°C for 20 seconds, annealing at

56°C for 25 seconds, and elongation at 68°C for 3 minutes. There was a final elongation at 68°C for 5 minutes. This was for the ITSFwd/GR-Rv amplicon and DL-Fw/ITS2.2 amplicon for *Proschizorhynchella sp.* (Figure 11, Table 1).

Paramonotus sp (ITS region):

The primers I used included ITSFwd, ITS2.2, ITS-BJFwd, STB5.8SFw, ITS-LZ, FOB-Rv (Table 1). I used ITSFwd (forward primer) and ITSLZ (reverse primer) to create one amplicon, and I used ITSBJFwd (forward primer) and ITS2.2 (reverse primer) to create the second amplicon (Figure 10, Table 1). They were overlaid to get two-fold consensus. STB5.8SFw and FOB-Rv were only used for sequencing primers. For sequencing, I used sequencing primers ITSFwd, FOB-Rv, and ITSLZ for the ITSFwd/ITSLZ amplicon, and I used ITS-BJFwd, STB5.8SFw, and ITS2.2 for the ITS-BJFwd/ITS2.2 amplicon (Table 1; Figure 12).

Proschizorhynchella sp. (ITS region):

The primers I used included ITSFwd, ITS2.2, DL-Fw, STB5.8SFw, and GR-Rv (Table 1). For ITS region, I first used ITSFwd as a forward primer and GR-Rv as a reverse primer to create an initial amplicon for the internal primers: ITSFwd and GR-Rv and DL-Rv. I also used DL-Fw as a forward primer and ITS2.2 as a reverse primer to create an initial amplicon for the internal primers: DL-Fw and ITS2.2 and STB5.8SFw (Figure 11). STB5.8SFw was only used for the sequencing primer (Figure 13). I could not put a reverse sequencing primer in the middle of the ITSFwd/GR-Rv (like I put the

FOB-Rv in the middle of ITSFwd/ITSLZ) because there are too many repeated segments in that region.

Sequence Alignment and Statistical Analyses:

Sequences were aligned with the MacVector Program. Consensus sequences were defined as areas of the sequence with at least two-fold overlap.

On the ABGD website, there are a variety different settings. First, there is P_{min} and P_{max}. The *P* value (not to be confused with the *p*-value) is defined as a prior limit to intraspecific diversity. "Interspecific diversity," in this context refers to the amount of diversity that is already found within a species. This is important in order to find a barcode gap because one needs to make sure that the amount of diversity that one is seeing is not just the normal amount of within-species diversity. P_{min} is the minimum amount of intraspecific diversity that one would expect to see, and P_{max} is the maximum amount of intraspecific diversity that one would expect to see. The second setting is "steps." Puillandre et al. (2012) do not explain what this is. Then there is *X*, which is the relative gap width. This refers to the size of the barcode gap. Next is Nb bins (for distance distribution). Puillandre et al. (2012) do not explain what this is either. Lastly, there were three different models of evolution to choose from Jukes-Cantor, Kimura, and Simple distance. The settings used were the default settings (P_{min}= 0.001, P_{max}=0.1, Steps= 10, X = 1.5, Nb bins= 20). I tried each of the distance parameters: Jukes-Cantor (JC69), Kimura (K80), and Simple distance. I tried running ABGD on each model to see if there was a difference. Previous studies into flatworms have used the default settings

for ABGD, so I used the default settings as well (Lemos et al. 2014, Rossi et al. 2015, Carbayo et al. 2017, Van Steenkiste 2018).

In order to determine within-species diversity, I used AMOVA (Analysis of Molecular Variance), as described by Excoffier et al. (1992), and I used the Arlequin program (Martinelli et al. 2007). AMOVA can use a hierarchical method by looking at diversity across regions, diversity across populations within regions, and diversity within populations. Fitzpatrick et al. (2009) found that hierarchical AMOVA needs at least 6 populations per region in order in order to result in *p*-values less than 0.05. Since I only had two populations per region, I used a non-hierarchical method, since there is no minimum population number needed to obtain a significant *p*-value. However, I could not find evidence that there was any minimum number of populations for the non-hierarchical AMOVA, which uses only one region. The non-hierarchical method looks only at diversity across populations and diversity within single populations (Excoffier et al. 1992, Martinelli et al. 2007).

Results:

Although I the goal was to obtain three sequences per specimen for each genetic marker at each collection site, I unfortunately was not able to do this for all of *Paramonotus sp.* ITS sequences (Table 2). This is because there was double sequencing for the majority of un-starved specimens because of food in the intestine of the specimens. Double sequencing is when DNA from more than one specimen is sequenced. This is caused by extraneous DNA, either from food in the intestine of the specimen or some sort of contamination of the sample. If there is any food in the intestine, say another flatworm or a diatom, Sanger sequencing will sequence both. If the amount of extraneous DNA is negligible, you may be able to align the sequences. I have done this on several occasions. However, sometimes, this is just not possible, and the contiguous files are completely unreadable. Often, I can see any extraneous DNA in the gel run, but it does not always show up, especially if the specimen amplicon and the extraneous DNA are the same base pair size.

As you can see from Table 2, there were no ITS sequences for *Paramonotus sp.* at Wrightsville Beach (WB). This is because 5 out of my seven specimens showed double sequences for my ITS primers. Also, there were only two consensus sequences for ITS region for *Paramonotus sp.* at Emerald Isle (EI) and North Topsail Beach (NTB). I had ten specimens for each of these sites, all un-starved. For both, I extracted DNA from 9 out of 10 of them. For my ITS primers, there was a significant amount of double sequencing from food in 7 out of 9 of them. The ITS region for *Paramonotus sp.* is very large, and if there was significant double sequencing in the middle of the sequence, then I

couldn't get a full consensus. I do have two more specimens, one from EI and one from NTB, but both of these are un-starved. Thus, they probably have food in their intestines, and so I decided not to try to sequence them.

Paramonotus sp. specimens were found at Iron Steamer Pier, Emerald Isle, North Topsail Beach, and Wrightsville Beach. *Proschizorhynchella sp.* was found at Emerald Isle, Wrightsville Beach, Oak Island, and Florida. Because of food in the intestines of the Oak Island samples, however, I was not able to get a three-fold sample for *Proschizorhynchella sp.* at Oak Island. Accordingly, I did not have the comparison between genetic markers and between species that I had planned to have.

Species Delimitation:

The 18S Gene:

For *Proschizorhynchella sp.*, all 18S consensus sequences were 100% identical. This includes specimens from Emerald Isle (EI), Wrightsville Beach (WB), and Florida (FL). No tests were done on these sequences, there was no genetic variation among them. For *Paramonotus sp.*, all 18S consensus sequences were also 100% identical. This includes specimens from Iron Steamer Pier (ISP), Emerald Isle (EI), North Topsail Beach (NTB), and Wrightsville Beach (WB). Again, no tests were done.

The 28S Gene:

For *Proschizorhynchella sp.*, all 28S consensus sequences were 100% identical, including specimens from Emerald Isle (EI), Wrightsville Beach (WB), and Florida (FL).

No tests were done on these sequences either, since was no genetic variation among them. For *Paramonotus sp.,* all 18S consensus sequences were also 100% identical. This includes specimens from Iron Steamer Pier (ISP), Emerald Isle (EI), North Topsail Beach (NTB), and Wrightsville Beach (WB). Again, no tests were done, as they were genetically identical.

The Cox-1 gene:

For *Proschizorhynchella sp.,* ABGD revealed two hypothetical species. Jukes-Cantor distance found 1 group, simple distance found one group, and Kimura distance found 2 groups. For Kimura, Group 1 (SP-X; Table 2, Light Green) included PSH608, PSH606, PSH607, PSH612, PSH604, and PSH603; all of these are from EI and WB collection sites (Table 2). Group 2 (SP-Y; Table 2, Pink) included PSH708, PSH705, and PSH706; these were from the FL collection site (Table 2). In other words, there were two hypothetical species detected: one from Onslow Bay sites and one from the Florida site. Figures 14 shows the distribution of these groups.

For *Paramonotus sp.,* Jukes-Cantor distance found three groups. Group 1 (SP-1; Table 2, Yellow) included STB528, STB527, STB537, STB514, STB510, STB505, STB536, and STB535. Group 2 (SP-2; Table 2, Blue) included STB521. Group 3 (SP-3; Table 2, Green) included STB605, STB606, and STB501 (Figure 15, Table 2). The distribution of the hypothetical species is shown in Figure 16. Kimura distance showed the same results. Simple distance only found one group, running only one partition.

ITS Region:

For *Proschizorhynchella sp.*, the results were a bit different from the cox-1 results. For Jukes-Cantor distance, 5 groups were found. Group 1 (SP-A; Table 2, Red) included PSH611; Group 2 (SP-B; Table 2, Purple) included PSH604, PSH605, PSH609, and PSH608; Group 3 (SP-C; Table 2, Brown) included PSH606; Group 4 (SP-D; Table 2, Orange) included PSH706 and PSH708; Group 5 (SP-E; Table 2, Gray) included PSH705 (Figure 16, Table 2). Kimura distance showed the same results. Simple distance found only one group. Figures 16 show the distribution of hypothetical species.

For *Paramonotus sp.*, all of the sequences I obtained were 100% identical. It is possible that a large sample size would show more variation, but for now it seems that the ITS region just evolves more slowly than the cox-1 gene. Another explanation is that the cox-1 results may have overestimated diversity for the ABGD tests.

Differences in Genetic Markers (AMOVA):

As previously stated, there was no variation across sites in either the 18S gene or the 28S for either *Paramonotus sp.* or *Proschizorhynchella sp.* For *Paramonotus sp.*, there was no variation in the ITS region either. However, there was some variation in the cox-1 gene for *Paramonotus sp.* A non-hierarchical AMOVA for the cox-1 gene for *Paramonotus sp.* found that 15.62% of variation came from among populations, and 84.38% of variation came from within populations (Table 3). This is not surprising, given the number of sympatric hypothetical species. However, these results were not significant (*p*-value= 0.51711+/-0.01583); the F_{ST} value was 0.63158 (Table 3).

For *Proschizorhynchella sp.*, I ran a non-hierarchical AMOVA on the sequences from EI, WB, and FL for the cox-1 gene and the ITS region. For the ITS region, I found that 0.00% of variation came from among populations while 100% of variation came from within populations. The F_{ST} value was 0.00, and the p-value was 0.79277+/- 0.01034 (Table 4). For the cox-1 region, I found that 63.16% of variation came from among populations while 36.84% of variation came from within populations. The F_{ST} value was 0.63158, and the p-value was 0.00391+/-0.00185 (Table 5).

Differences between Morphospecies:

There were only two sites where both *Paramonotus sp.* and *Proschizorhynchella sp.* were present: Emerald Isle (EI) and Wrightsville Beach (WB). Thus, I ran two non-hierarchical AMOVAs for the cox-1 gene: one for *Paramonotus sp.* for the EI and WB populations and one for *Proschizorhynchella sp.* for the EI and WB populations. For *Proschizorhynchella sp.*, I found that 21.43% of variation came from among populations and that 78.57% of variation came from within populations. The F_{ST} value was 0.21429, but the p-value was 0.20528+/- 0.01417 (Table 6). For *Paramonotus sp.*, I found that 0.00% of variation came from among populations while 100% of variation came from within populations. The F_{ST} value was 0.00, and the p-value was 1.000 (Table 7).

Discussion:

Differences between Genetic Markers:

For *Paramonotus sp.*, there was no variation in 18S gene, 28S gene, or ITS region consensus sequences. Only the cox-1 gene consensus sequences showed any genetic variation across populations for *Paramonotus sp.*, as is shown by the hypothetical species detected by ABGD (Figure 15, Table 3).

For *Proschizorhynchella sp.*, there was no variation in the 18S gene consensus sequences or the 28S gene consensus sequences either. The ITS region sequences showed some variation across populations, as seen by the hypothetical species that were detected by ABGD (Figures 16). However, AMOVA did not find a significant amount of variation across populations for the ITS region (Table 4). This is likely due to a small sample size. There was a significant amount of variation across population for the cox-1 gene sequences, as shown by AMOVA (Table 5). ABGD also found two hypothetical species for the cox-1 gene: one in Onslow Bay and one in Florida (Figure 14).

This varies somewhat from my hypothesis that the 18S gene would have the least amount of genetic variation across populations, followed by the 28S gene, the ITS region, and the cox-1 gene (which I hypothesized would have the most amount of variation). I would venture that the reason both 18S gene and 28S gene showed no variation is because my collection sites were not far apart enough geographically. Van Steenkiste et al (2018) found that 18S gene was ineffective at delimiting cryptic species for a genus of

meiofaunal flatworm. Since I did not find any genetic variation in the 28S gene, this provides evidence that 28S is not very effective at detecting cryptic speciation among meiofaunal flatworms. However, one should keep in mind that the reason that I did not see as much variation in the 18S gene and the 28S gene in my study is that my collection sites were relatively close geographically, as opposed to the study by Van Steenkiste et al. (2018) which included samples over a much larger geographic area, ranging from Europe to North America.

For *Paramonotus sp.*, ITS region sequences did not show any variation, and there are two possible explanations for this. First, my sample size might have been too small; perhaps more samples would show sequences with a bit more variation. The second explanation is that my collection sites were not separated far enough geographically. *Paramonotus sp.* has not been found outside Onslow Bay, but if specimens were found outside of Onslow Bay, it would be worth investigating those populations in future studies.

For *Proschizorhynchella sp*., there was some variation among the ITS region sequences, but it was not as high as it was for cox-1 gene. Although a non-hierarchical AMOVA at this sample size is not that powerful, the cox-1 gene did show a significant amount of variation across populations.

I could not find evidence from previous studies that would indicate that different species or taxa would have large differences in mutation rates for the ITS region for metazoans. This would indicate that any differences in evolution are not due to

differences in mutation rates but other evolutionary mechanisms, such as gene flow. However, Kay et al. (2006) did a study to find a universal substitution rate for the ITS region of angiosperms and found that it is unlikely that there is a universal substitution rate for angiosperms. This suggests that the substation rate may be different for *Paramonotus sp.* and *Proschizorhynchella sp.*, but it may be a stretch to draw any definite conclusions through comparing angiosperms to platyhelminths.

The Occurrence of Cryptic Species:

For *Proschizorhynchella sp.*, all 18S and 28S sequences were 100% identical. At the same time, however, the cox-1 gene revealed two hypothetical species: one in Onslow Bay and one in Florida (Figure 14). This is not too surprising, since the Florida site is about 500 miles from Onslow Bay. The ITS region revealed five hypothetical species (Figures 16). I named these SP-A (group 1), SP-B (group 2), SP-C (group 3), SP-D (group 4), and SP-E (group 5). SP-A is only found in WB, SP-B is found in EI and WB, SP-C is only found in EI, SP-D is only found in FL, and SP-E is also only found in FL. SP-A, SP-B, and SP-C are only found in Onslow Bay, while SP-D and SP-E are only found in Florida (at least in this study). This supports the idea that the Florida population has its own genetic identity, giving credence to the supposition that the Florida population of *Proschizorhynchella sp.* is its own species; hereafter it will be referred to as *Proschizorhynchella sp. nov.*

For *Paramonotus sp.*, all 18S and 28S sequences were also 100% identical. The cox-1 gene revealed three hypothetical species (Figure 15). However, all of the ITS

sequences I collected were 100% identical, which suggests that all of these *Paramonotus sp*. consist of one species. Thus, the prior maximal distance may have been set too low. In other words, the variation we are seeing here for cox-1 gene may just be intraspecies variation.

There are not many studies on meiofaunal flatworms that focus on the efficacy of different genetic markers for the detection of cryptic species. Van Steenkiste et al. (2018) found sympatric species among the genus *Astrotorhynchus*, but most of these were pseudo-cryptic (species identified *a posteri*) and could be distinguished from each other based on the stylet. Additionally, they found evidence that the cox-1 gene might be overestimating species diversity, but this may be due to the fact that they had a lower sample size for cox-1 sequences than for 18S or 28S for the multi-gene species delimitation (Van Steenkiste et al. 2018). They had a larger sample size and ran more tests, but I provide evidence that 28S is not effective in the face of ITS region and the cox-1 gene. Also, it is worth noting that they had obtained a larger fragment of the cox-1 gene than I did.

One may wonder if it is appropriate to use ABGD at default settings and with this small a sample size, but previous studies have done the same with non-meiofaunal free-living flatworms with a comparable sample size. Lemos et al (2014) was looking at delineating two new species of triclads using morphological and molecular techniques and used ABGD at the default settings. They used cox-1 and ITS-1 as genetic markers, and they had ten sequences for cox-1 and 16 for the ITS-1. Rossi et al (2015) found two new species of Geoplaninae using morphological and molecular techniques. They used

cox-1 for a genetic marker and default settings for ABGD with 9 samples. Carbayo et al (2017) found new cryptic species in the genus *Choeradoplana* by using a much larger sample size but still using the default settings for 18S, 28S, cox-1, and ITS-1.

Detecting a Genetic Gradient:

I did not find a genetic gradient *per se*, but there was some genetic variation across populations for both morphospecies. Although it is apparent that both of these morphospecies were monophyletic, I did not find evidence that there was any gradient where separate cryptic species slowly became a different species over a geographic distance. This is could be because there were not enough collection sites to see a gradient. Additionally, it may not be possible to see a good gradient using these markers. They are usually used for measuring diversity because they do not change very much.

For *Paramonotus sp.*, there was a slight amount of genetic divergence across populations, as can be seen by the number of hypothetical species. For *Proschizorhynchella sp.*, there was a small amount of variation across populations, as can be seen by the amount of hypothetical species. AMOVA showed a significant amount of variation across populations.

Is there a difference in variation between the two morphospecies?

Although AMOVA did not show a significant amount of variation across populations for either, there was numerically more genetic variation across a geographic distance for *Proschizorhynchella sp.* than for *Paramonotus sp.* The AMOVA tests I am referring to are in Table 6 and Table 7, where only the populations from Wrightsville

Beach and Emerald Isle were incorporated. Thus, the populations in these tests were from the same two sites. I did this because I wanted to look at populations of the two morphospecies across the same geographic distance.

However, neither of these tests showed a significant amount of variation, so this may not be meaningful. Also note that I could not test the ITS region marker because I could not obtain any ITS region sequences from WB for *Paramonotus sp.* In addition, a larger sample size may result in significant differences. Since there were only two collection sites and only the cox-1 genetic marker was tested, it is not surprising that I did not reap significant results here.

Despite this, there was numerically more genetic variation across populations for the stationary morphospecies (*Proschizorhynchella sp.*), and this lends some credence to the idea that the three adhesive belts of *Proschizorhynchella sp.* allow it to resist being swept into the water column, thus reducing gene flow between populations. It also gives some evidence for the idea that the reason so many meiofaunal species seem to be cosmopolitan is simply because they are able to maintain gene flow if they allow themselves to be swept up into the water column, which in turn carries them some distance. Obviously, future studies need to expand upon this topic by increasing the sample size.

Concluding Remarks:

There does not seem to be a silver bullet for detecting cryptic species among meiofaunal flatworms. Using Automatic Barcode Gap Discovery, my findings indicate

that the best path would be to sequence both the cox-1 gene and the ITS region. Then, one can determine the optimal prior maximal distance for ABGD. Although I was only able to sequence a fragment of the cox-1 gene, this is the first study that I am aware of that uses the entire ITS region as a genetic marker for free-living flatworms. My findings also suggest that 18S and 28S may not be effective at detecting cryptic speciation among meiofaunal flatworms. Studies that have only used 18S or 28S to find cryptic species of meiofaunal flatworms may have underestimated their diversity. Future studies can expand upon this by using a larger sample size, more collection sites, and multi-locus gene delimitation.

It seems that *Proschizorhynchella sp.* can be separated into two separate biological species: one from the Onslow Bay populations and one from the Florida population (*Proschizorhynchella sp. nov.*). *Paramonotus sp.*, however, does appear to constitute a single biological species, at least for the populations in this study. This suggests that although geographic distance can lead to cryptic speciation among meiofaunal flatworms, the geographical size of Onslow Bay is not large enough to have different cryptic species. My findings do indicate that there were genetic variations across population, although there was not a genetic gradient *per se*. This may be because the markers I have chosen have a low mutation rate. While this makes them good at identifying species, it may not be the best way to find a genetic gradient.

Although everything is not everywhere, I think that cryptic speciation is only one piece of the puzzle that is the meiofaunal paradox. Although I did find evidence of cryptic species among my flatworm morphospecies, I found less than I would expect if

actively crawling through sediment were the only mode of transportation. Gene flow must be maintained by another mechanism, and this mechanism is probably their ability to be carried passively by the water column. Hagerman and Rieger (1981) found that meiofauna were routinely suspended in the water column in Bogue Sound, NC. They also found evidence that residual currents in Bogue Sound may be capable of carrying meiofauna that are suspended in the water column up to 10 km per day (Hagerman and Rieger 1981). My study provides evidence that passive transport along the water column in tandem with the occurrence of cryptic speciation may explain the meiofaunal paradox, at least for flatworms.

This study is novel for the fact that I was able to use the entire ITS region as a genetic marker for free-living flatworms. I am not the first to sequence the entire ITS region for free-living flatworms, but I am one of the first. There has only been one published study that has sequenced the entire ITS region for a free-living flatworm and this was only for one species (Scarpa et al. 2016b) Also, I am not aware of another study that uses two morphospecies from two different orders of flatworm to look at genetic differentiation and the occurrence of cryptic speciation.

Future studies should use the genetic markers that I have used and obtain a larger sample size and more collection sites over a larger geographic range. If one wishes to observe a genetic gradient, one should use more variable markers, such as SNPs. I could not find any studies that have used SNPs (single nucleotide polymorphisms) as genetic markers for free-living flatworms. Most importantly, one should starve meiofaunal flatworms overnight, as I have found that even a small amount of extraneous DNA from

food in the intestine can cause double sequencing. This can be seen by the fact that I was only able to sequence 7 specimens of *Paramonotus sp.* for the ITS region. Using Phusion Taq is preferable, but it will not remove extraneous DNA, only provide fewer sequencing errors.

Figures and Tables:

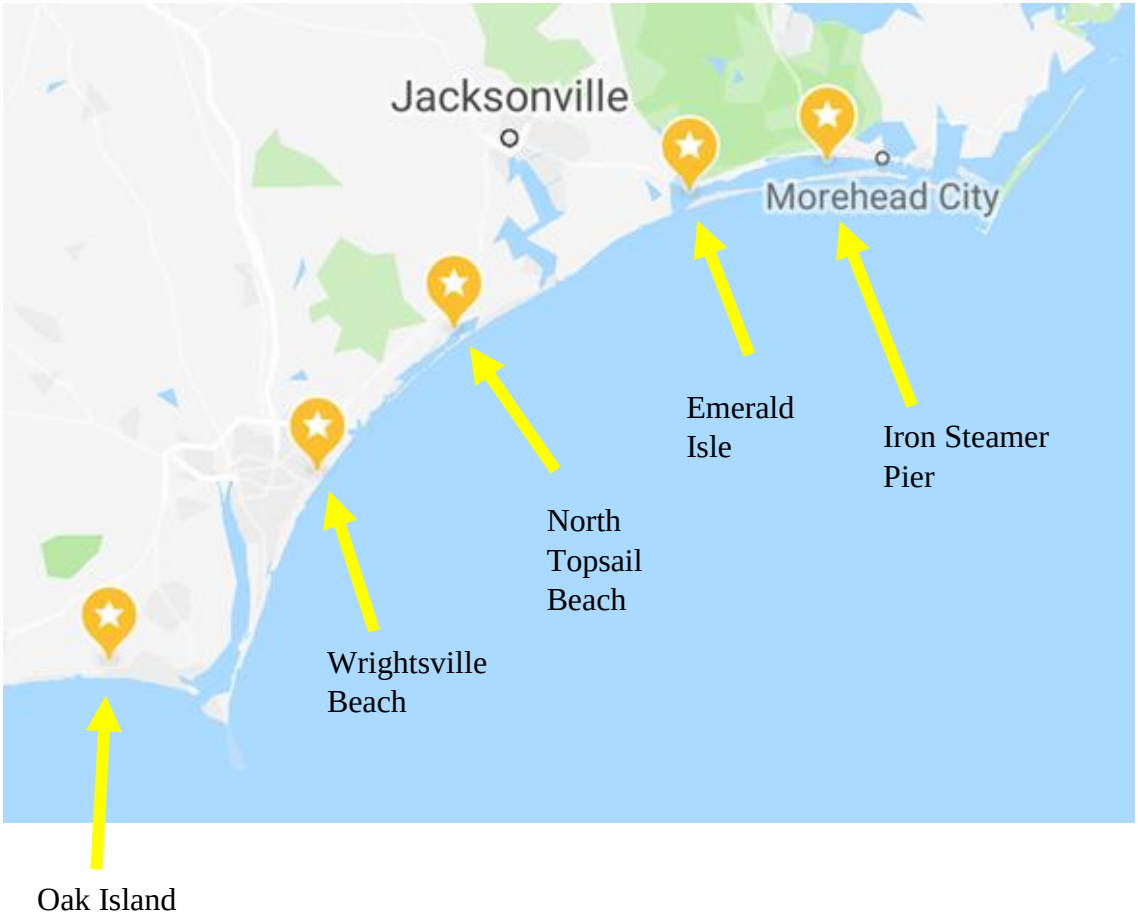

Figure 1: Map of Sampling Locations in North Carolina; Image Provided by Google Maps

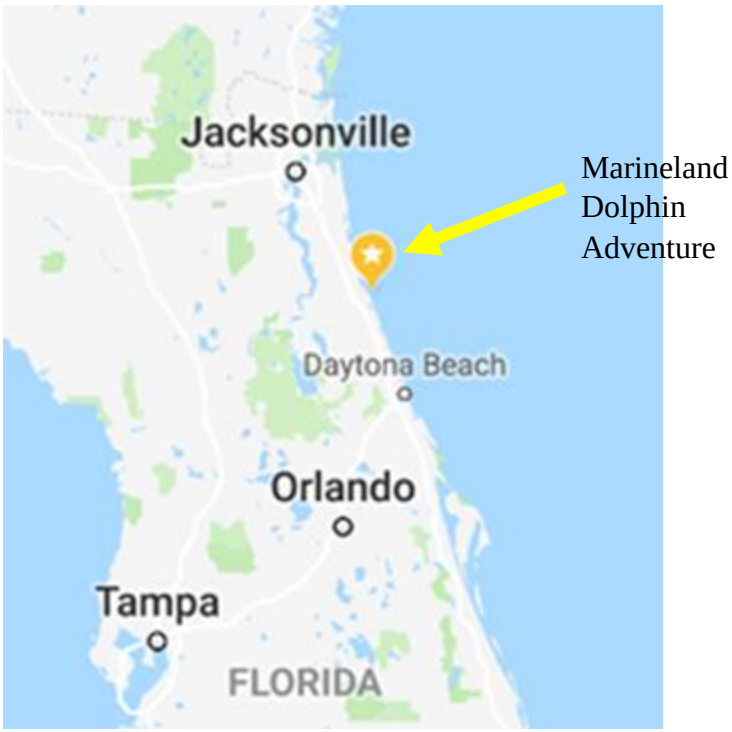

Figure 2: Map of sampling location in Florida; image provided by Google Maps

Table 1: List of primers and their sources

Primer Name	Sequence	Source
TimA	AMCTGGTTGATCCTGCCAG	Noren and Jondelius 1999
TimB	TGATCCATCTGCAGGTTCACCT	Noren and Jondelius 1999
600F	GGTGCCAGCAGCCGCGGT	Willems et al. 2006
600R	ACCGCGGCTGCTGGCACC	Willems et al. 2006
1100F	CAGAGGTTCGAAGACGATC	Noren and Jondelius 1999
1100R	GATCGTCTTCGAACCTCTG	Noren and Jondelius 1999
HNRV	AACCTTGTTACGACTTTTACTTCCTC	Maghsoud et al. 2014
LSU5	TAGGTCGACCCGCTGAAYTTA	Littlewood et al. 2000
LSUD6-3	GGAACCCTTCTCCACTTCAGTC	Littlewood et al. 2000
Poly28SF1	TGAAAAGAACTTTGAAGAGAGAGT	Tessens et al. 2014
Poly28SR2	TGCTACTRCCACCAAGATCTRCWCC	Tessens et al. 2014
MICOIintF	GGWACWGGWTGAACWGTAYCCYCC	Leray et al. 2013
jgHCO2198	TAACYTCGGRTGCCRAARAAYCA	Leray et al. 2013
ITSFwd	TGCCCTTTGTACACACCGC	This study
ITS2.2	CCTGGTTAGTTTCTTTTCCTCCGC	Goggin and Newman 1996
ITS-BJFwd	GTGGTCTCATCCAACAGAGAGC	This study
STB5.8SFw	GTCGATGAAGAGCGCAGC	This study
STB5.8SRv	GTCTGCGCTCTTCATCGAC	This study
ITS-LZ	CATCTAACCAACCGCTCCTGG	This study
FOB-Rv	TTTAGACTTCTCTGTTGCCGGAGAA	This study
DL-Fw	CCCAAAAACGTAATCCCTACACGGTAT	This study
GR-Rv	CATTTGCACGTTATTTCCACGAGCG	This study

Figure 3: This is the original amplicon for 18S, where the primer set is TimA (forward primer) and TimB (reverse primer).

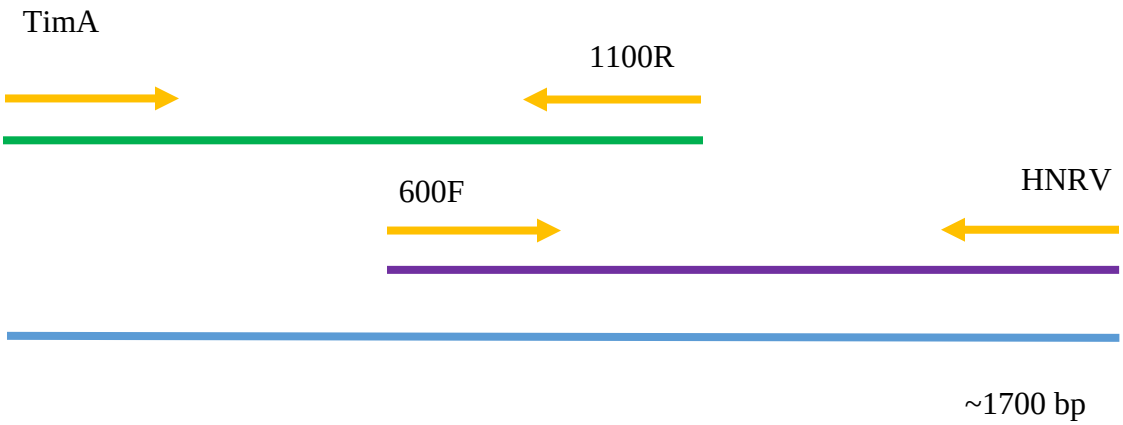

Figure 4: The internal amplicons for 18S. The upstream amplicon has the primer set of TimA (forward primer) and 1100R (reverse primer). The downstream amplicon has the primer set of 600F (forward primer) and HNRV (reverse primer).

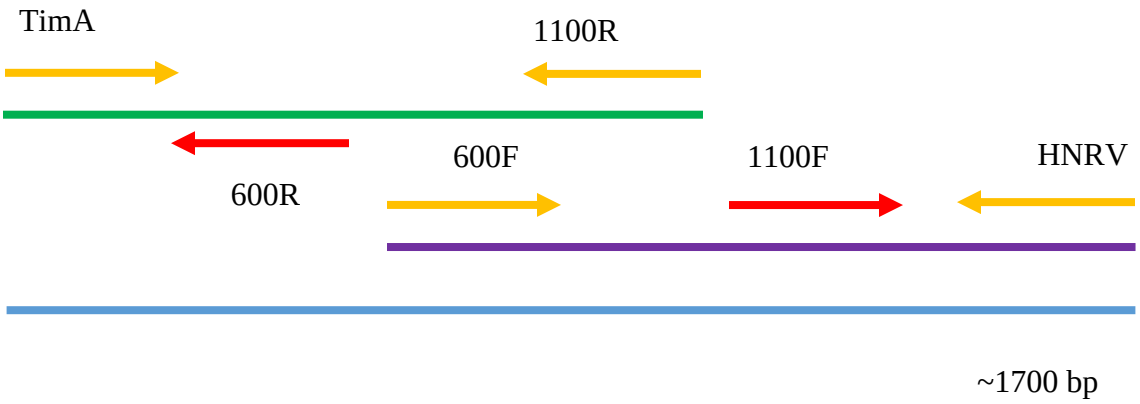

Figure 5: These are the sequencing primers for 18S. For the TimA/1100R amplicon, I used TimA (forward primer), 1100R (reverse primer), and 600R (reverse primer). For the 600F/HNRV amplicon, I used 600F (forward primer), 1100F (forward primer), and HNRV (reverse primer).

Figure 6: This is the original amplicon for 28S, where the primer set is LSU5 (forward primer) and LSUD6-3 (reverse primer).

Figure 7: Internal amplicon for 28S, where the primer set is Poly28SF1 (forward primer) and Poly28SR2 (reverse primer).

Figure 8: Sequencing primers for 28S. For the original amplicon, LSU5 (forward primer) and LSUD6-3 (reverse primer) were used. For the internal amplicon, Poly28SF1 (forward primer) and Poly28SR2 (reverse primer) were used.

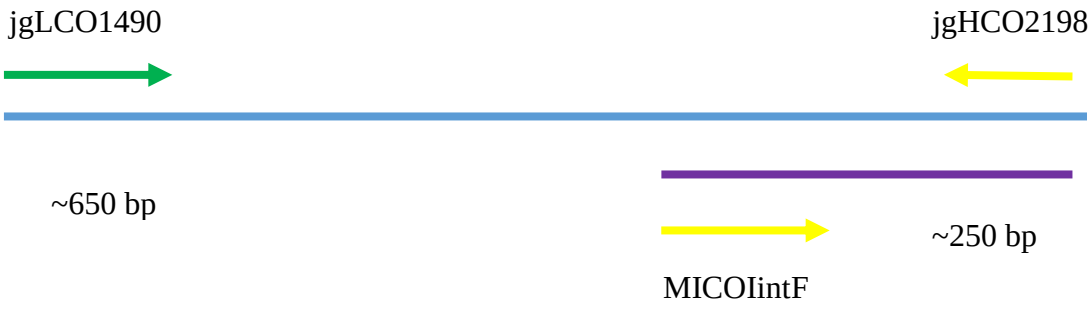

Figure 9: This shows where the fragment that I used falls within the "Folmer" region. As you can see, the "Leray" fragment is at the tail end of the "Folmer" region. I did not use jgLCO1490. The primer set I used was MICOIintF (forward primer) and jgHCO2198 (reverse primer) sequencing primers are the same as the PCR primers.

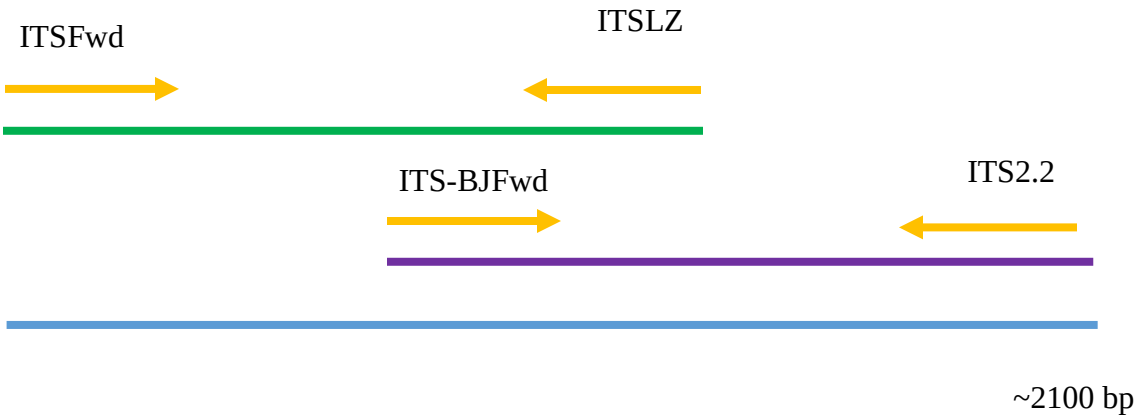

Figure 10: This is the primer set for the ITS region for *Paramonotus sp*. Two separate PCR reactions were run. The upstream amplicon used ITSFwd (forward primer) and ITSLZ (reverse primer). The downstream amplicon used ITS-BJFwd (forward primer) and ITS2.2 (reverse primer).

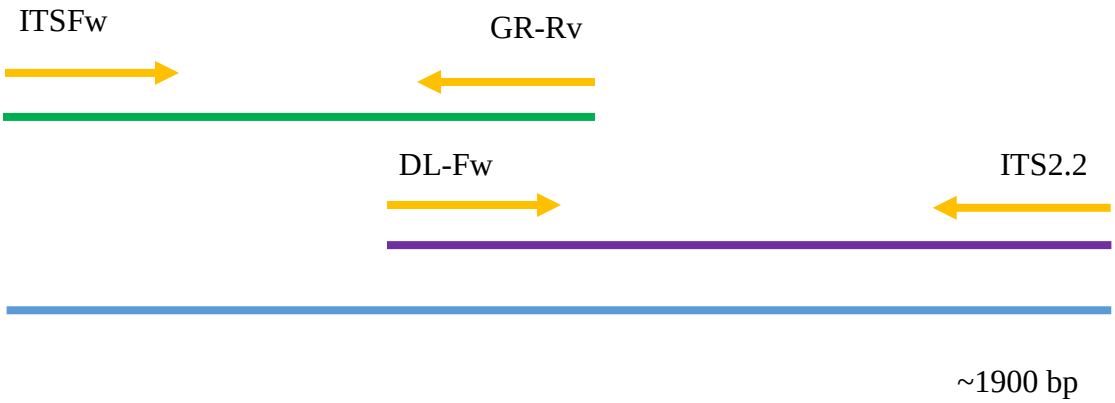

Figure 11: This is the primer set for the ITS region for *Proschizorhynchella sp.* Two separate PCR reactions were run. The upstream amplicon used ITSFwd (forward primer) and GR-Rv (reverse primer). The downstream amplicon used DL-Fw (forward primer) and ITS2.2 (reverse primer).

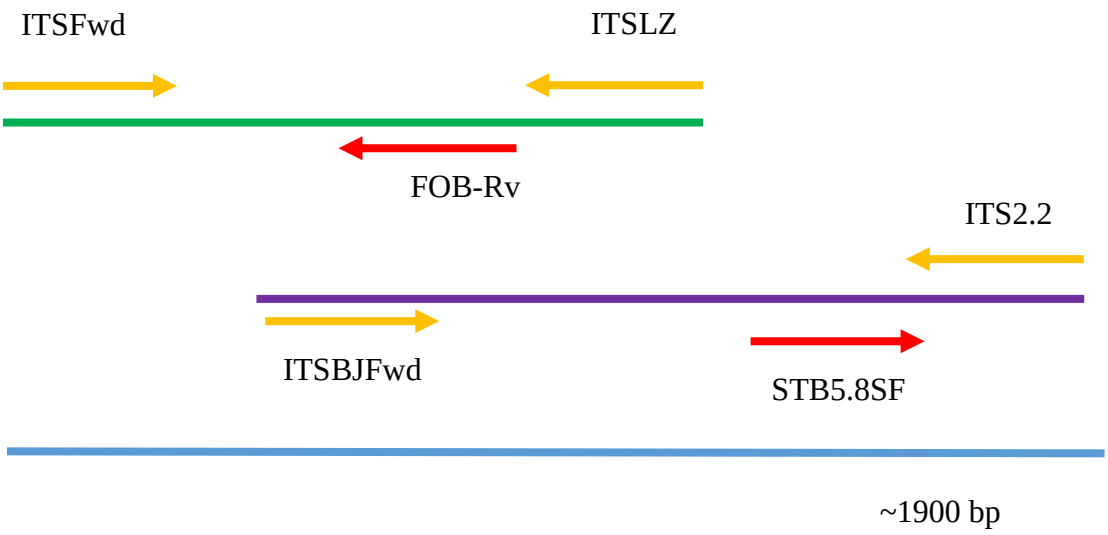

Figure 12: Sequencing primers used for the ITS region for *Paramonotus sp.* The upstream amplicon used ITS-Fwd (reverse primer), ITS-LZ (reverse primer), and FOB-Rv (reverse primer). The downstream amplicon used ITS-BJFwd (forward primer), STB5.8SFw (forward primer), and ITS2.2 (reverse primer).

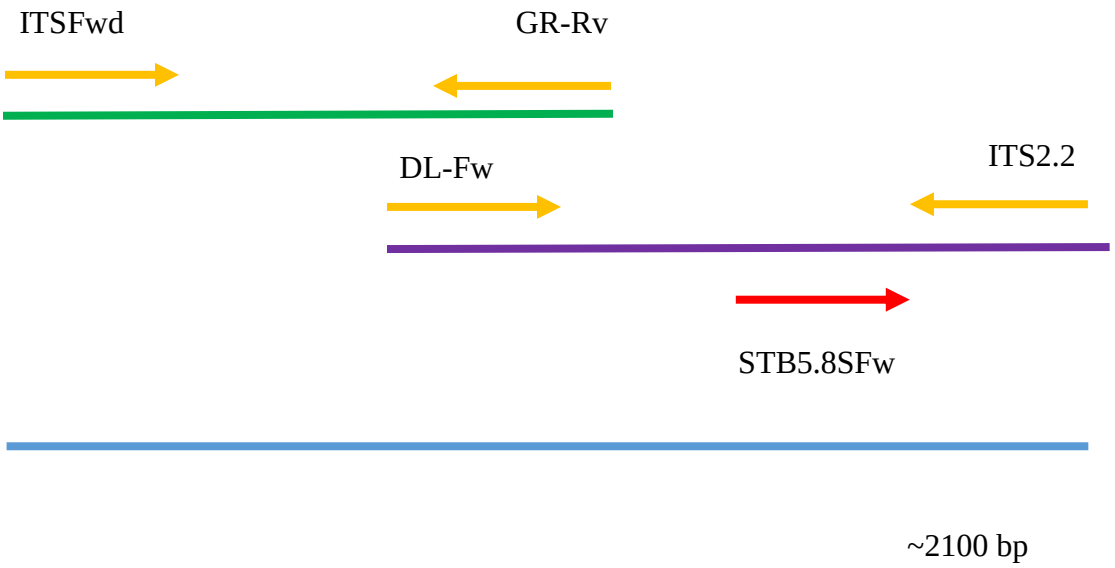

Figure 13: The sequencing primers for the ITS region for *Proschizorhynchella sp.* For the upstream amplicon, ITSFwd (forward primer) and GR-Rv (reverse primer) were used. For the downstream amplicon, DL-Fw (forward primer), STB5.8SFw (forward primer), and ITS2.2 (reverse primer).

Table 2: Specimens and sequences for each genetic maker for each putative species. "STB" refers to *Paramonotus sp.* and "PSH" refers to *Proschizorhynchella sp.* "ISP" stands for Iron Steamer Pier, "EI" stands for the Emerald Isle site, "WB" stands for Wrightsville Beach, "NTB" stands for North Topsail Beach, and "FL" stands for the Florida site. The different hypothetical species detected by Automatic Barcode Gap Discovery are color-coded. SP-X: light green; SP-Y: pink; SP-1: yellow; SP-2: blue; SP-3: green; SP-A: red; SP-B: purple; SP-C: brown; SP-D: orange; SP-E: gray

	Paramonotus				Proschizorhynchella		
	ISP	EI	NTB	WB	EI	WB	FL
18S	STB511	STB521	STB501	STB531	PSH606	PSH601	PSH706
	STB514	STB525	STB502	STB532	PSH607	PSH604	PSH705
	STB606	STB526	STB503	STB533	PSH608	PSH605	PSH708
28S	STB511	STB521	STB501	STB531	PSH606	PSH601	PSH706
	STB514	STB525	STB502	STB533	PSH607	PSH604	PSH709
	STB515	STB523	STB503	STB536	PSH608	PSH611	PSH704
COI	STB514	STB521	STB501	STB536	PSH608	PSH604	PSH706
	STB606	STB527	STB510	STB537	PSH606	PSH603	PSH708
	STB605	STB528	STB505	STB535	PSH607	PSH612	PSH705
ITS	STB607	STB521	STB501	N/A	PSH606	PSH604	PSH705
	STB511	STB527	N/A	N/A	PSH608	PSH605	PSH706
	STB608	N/A	STB502	N/A	PSH609	PSH611	PSH708

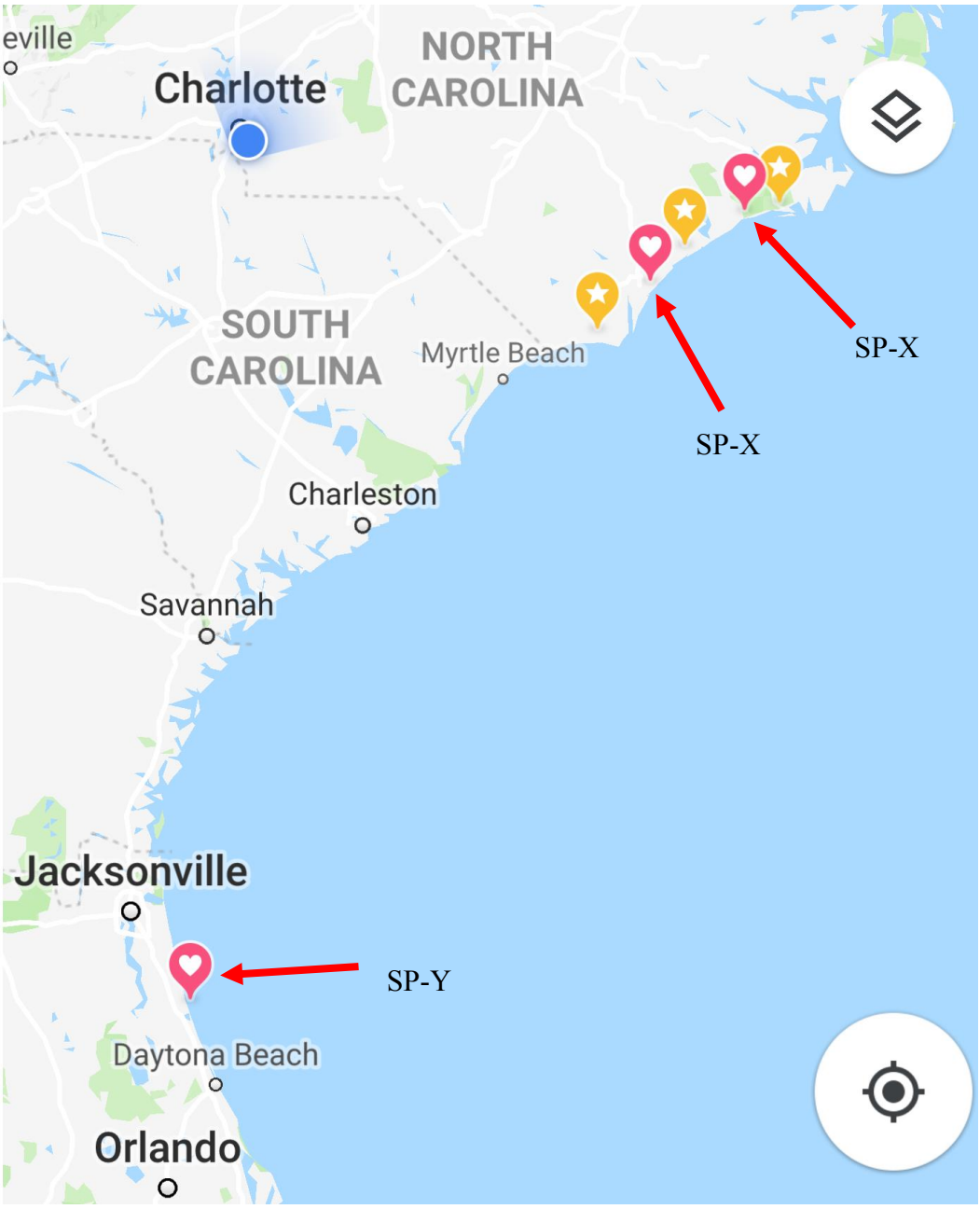

Figure 14: Distribution of hypothetical species detected by ABGD for the cox-1 gene for *Proschizorhynchella sp.* Group 1→SP-X (EI, WB); Group 2→ SP-Y (FL); Image Provided by Google Maps. The hearts indicate collection sites where *Proschizorhynchella sp.* was found.

Figure 15: Distribution of hypothetical species detected by ABGD for the cox-1 gene for *Paramonotus sp.* Group 1= SP1; Group 2= SP2; Group 3=SP3; Image Provided by Google Maps

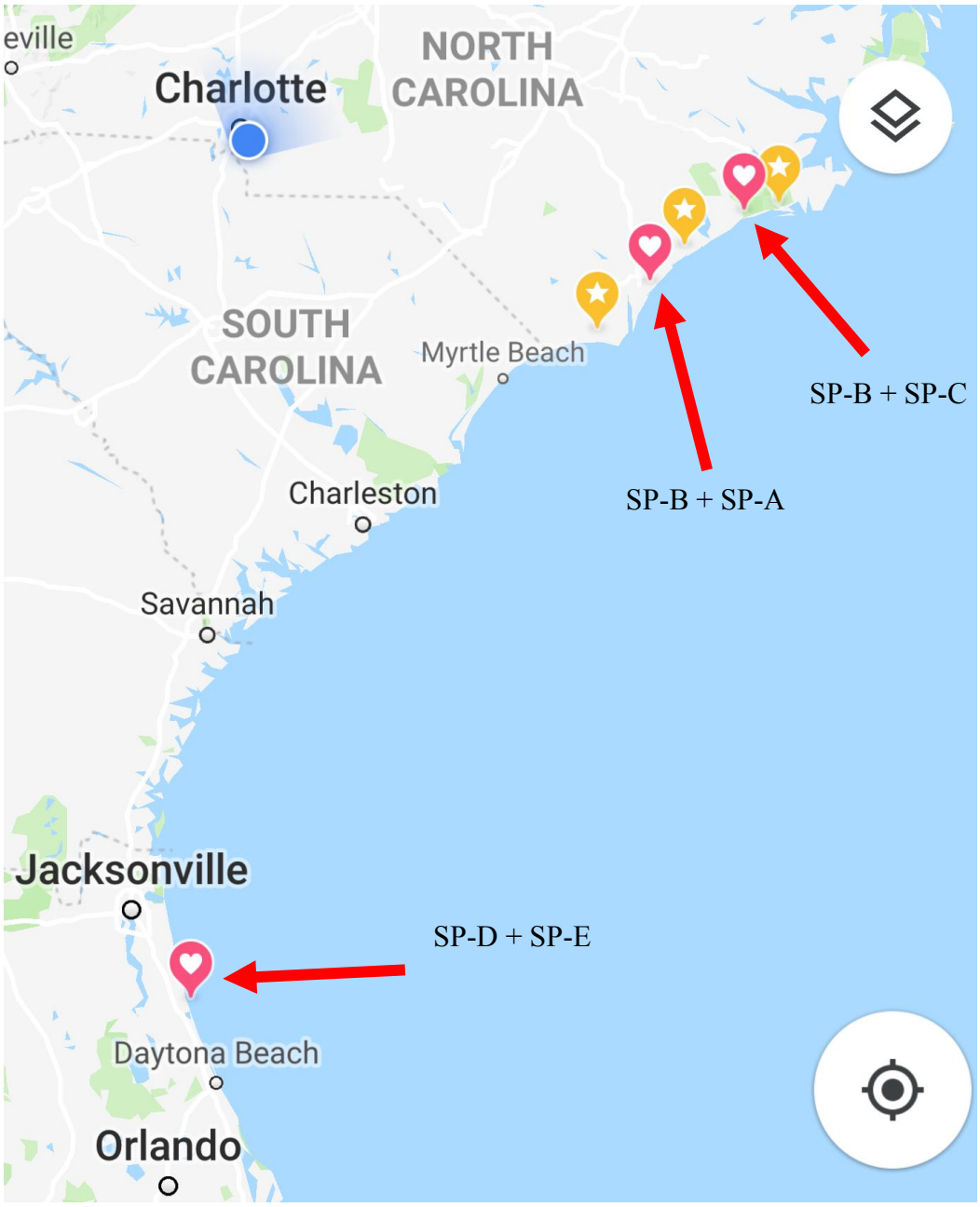

Figure 16: Distribution of hypothetical species detected by ABGD for the ITS region for *Proschizorhynchella sp.* Group 1= SP-A; Group 2= SP-B; Group 3= SP-C, Group 4= SP-D, Group 5= SP-E. Image Provided by Google Maps. The hearts indicate collection sites where *Proschizorhynchella sp.* was found.

Table 3: AMOVA results for the cox-1 gene for *Paramonotus sp.* from all sites (ISP, NTB, EI, WB).

Source of Variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)	p-value
Among populations	2	19.111	2.66667 Va	0	
Within populations	6	9.333	1.55556 Vb	100	
Total	8	28.444	4.22222		0.00391+/-0.00185
Fixation index	0.63158				

Table 4: AMOVA results for the ITS region for *Proschizorhynchella sp.* from all sites (EI, WB, FL).

Source of Variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)	p-value
Among populations	2	1.111	0.00 Va	0	
Within populations	6	3.333	0.55556 Vb	100	
Total	8	4.444	0.55556		0.79277+/-0.01034
Fixation index	0				

Table 5: AMOVA results for the cox-1 gene for *Proschizorhynchella sp.* from all sites (EI, WB, FL).

Source of Variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)	p-value
Among populations	2	19.111	2.66667 Va	63.16	
Within populations	6	9.333	1.55556 Vb	36.84	
Total	8	28.444	4.22222		0.00391+/- 0.00185
Fixation index	0.63158				

Table 6: AMOVA results for the cox-1 gene for *Proschizorhynchella sp.* from two sites (EI and WB).

Source of Variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)	p-value
Among populations	1	3.333	0.5 Va	21.43	
Within populations	4	7.333	1.83333 Vb	78.57	
Total	5	10.667	2.33333		0.20528+/- 0.01417
Fixation index	0.21429				

Table 7: AMOVA results for the cox-1 gene for *Paramonotus sp.* from two sites (EI and WB).

Source of Variation	Degrees of freedom	Sum of squares	Variance components	Variation (%)	p-value
Among populations	1	0.167	0.00 Va	0	
Within populations	4	0.667	0.16667 Vb	100	
Total	5	0.833	0.16667		1
Fixation index	0				

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Appendices:

Appendix A: Extracting DNA from specimen with Qiagen kit:

1) Withdraw as much ethanol as possible
2) Evaporate off ethanol in heating block for 20 minutes for 50°C
3) Add 180 uL of ATL buffer
4) Add 20uL of proteinase K
5) Incubate for 2 hours at 50°C in heating block
6) Add 200 uL of AL buffer
7) Add 200 uL of 100% ethanol
8) Load all 600 uL onto Qiagen kit column
9) Spin at 8K for one minute
10) Transfer column to new collecting tube
11) Wash with 500 uL of AW1 buffer
12) Spin at 8K for 1 minute and transfer column to a new collecting tube
13) Wash with 500 uL AW2 buffer
14) Spin at 12K for one minute and transfer to new collecting tube
15) Spin at 12K for another two minutes
16) Transfer to sterile Eppendorf tube
17) Add 30 uL of AE buffer
18) Let stand at room temperature for 5-10 minutes
19) Spin at 8K for one minute

Appendix B: Using MinElute Kit:

1) Remove MinElute spin columns from the fridge.
2) Add 5 volumes of PB buffer to 1 volume of PCR reaction
    a. Mix in the PCR tube
3) Add all onto MinElute spin column
4) Spin for 1 minute at 13k in the centrifuge
5) Remove column and place in a new catch tube
6) Add 750 µL of PE buffer to column and spin for 1 minute
7) Place column in new catch tube and spin for 2 minutes
8) Place column in an Eppendorf tube
9) Add 15 µL of EB buffer to column
10) Let sit for 5-10 minutes
11) Spin for 1 minute

Appendix C: This table documents every sequence that I used, including the date it was sequenced, the primer used, the DNA polymerase used, the concentration of the specimen DNA extraction, and the location in my lab notebooks.

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB501	6.6	TimA	ADA594	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	1100R	ADA595	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	600R	ADA596	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	600F	ADA597	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	HNRV	ADA598	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	1100F	ADA599	18S	6/2/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	LSU5	ACY035	28S	6/6/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	LSUD6-3	ACY036	28S	6/6/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	Poly28SF1	ACY039	28S	6/6/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	Poly28SR2	ACY040	28S	6/6/2016	Bk 2, p. 5	NTB	taq	NEB18S	20
Paramonotus sp.	STB501	6.6	MICOIintF	APN089	COI	10/12/2017	Bk. 5, p. 115	NTB	taq	LERAY5	20
Paramonotus sp.	STB501	6.6	jgHCO2198	APN090	COI	10/12/2017	Bk. 5, p. 115	NTB	taq	LERAY5	20
Paramonotus sp.	STB501	6.6	FOB-Rv	ATW661	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB501	6.6	ITSFwd	ATW659	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB501	6.6	ITS-LZ	ATW660	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB501	6.6	ITS-BJFwd	ATW662	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB501	6.6	ITS2.2	ATW663	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB501	6.6	STB5.8SFw	ATW664	ITS	4/8/2018	Bk. 8, p. 7	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB502	5.5	TimA	ADA953	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	1100R	ADA954	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	600R	ADA963	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	600F	ADA957	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	1100F	ADA961	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	HNRV	ADA958	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	LSU5	ADA965	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	LSUD6-3	ADA966	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	Poly28SF1	ADA969	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB502	5.5	Poly28SR2	ADA970	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	TimA	ADA955	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	600R	ADA964	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	1100R	ADA956	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB503	9.3	600F	ADA959	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	HNRV	ADA960	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	1100F	ADA962	18S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	LSU5	ADA967	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	LSUD6-3	ADA968	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	Poly28SF1	ADA971	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB503	9.3	Poly28SR2	ADA972	28S	2/17/2017	Bk 3, p. 22	NTB	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	TimA	ADA894	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	600R	ADA899	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	1100R	ADA895	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	600F	ADA901	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	HNRV	ADA902	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	1100F	ADA903	18S	7/12/2016	Bk 2, p. 57	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	LSU5	ADA909	28S	7/26/2016	Bk 2, p. 70	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	LSUD6-3	ADA910	28S	7/26/2016	Bk 2, p. 70	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	Poly28SF1	ADA913	28S	7/26/2016	Bk 2, p. 70	ISP	taq	NEB18S	20
Paramonotus sp.	STB511	5.1	Poly28SR2	ADA914	28S	7/26/2016	Bk 2, p. 70	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	MICOIintF	APN095	COI	10/12/2017	Bk. 5, p. 115	ISP	taq	LERAY5	20
Paramonotus sp.	STB514	9.9	jgHCO2198	APN096	COI	10/12/2017	Bk. 5, p. 115	ISP	taq	LERAY5	20
Paramonotus sp.	STB514	9.9	TimA	ADA997	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	600R	ADB007	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	1100R	ADA998	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	600F	ADB001	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	HNRV	ADB002	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	1100F	ADB003	18S	3/26/2017	Bk 3, p. 65	ISP	taq	NEB18S	20
Paramonotus sp.	STB514	9.9	LSU5	ADB009	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB514	9.9	LSUD6-3	ADB010	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB514	9.9	Poly28SR2	ADB013	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB514	9.9	Poly28SF1	ADB014	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB515	17.1	LSU5	ADB011	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB515	17.1	LSUD6-3	ADB012	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB515	17.1	Poly28SR2	ADB017	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB515	17.1	Poly28SF1	ADB034	28S	3/26/2017	Bk 3, p. 65	ISP	phusion	JS3TIMPF	20
Paramonotus sp.	STB605	1.2	MICOIintF	AHM813	COI	6/22/2017	Bk. 4, p. 100	ISP	taq	LERAY3	20
Paramonotus sp.	STB605	1.2	jgHCO2198	AHM814	COI	6/22/2017	Bk. 4, p. 100	ISP	taq	LERAY3	20
Paramonotus sp.	STB606	4.6	MICOIintF	AHM815	COI	6/22/2017	Bk. 4, p. 100	ISP	taq	LERAY3	20
Paramonotus sp.	STB606	4.6	jgHCO2198	AHM816	COI	6/22/2017	Bk. 4, p. 100	ISP	taq	LERAY3	20
Paramonotus sp.	STB521	5.9	600R	ADA907	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	TimA	ADA897	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	1100R	ADA898	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	600F	ADA904	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	HNRV	ADA905	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	1100F	ADA906	18S	7/12/2016	Bk 2, p. 57	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	LSU5	ADA911	28S	7/26/2016	Bk 2, p. 20	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	LSUD6-3	ADA912	28S	7/26/2016	Bk 2, p. 20	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	Poly28SF1	ADA915	28S	7/26/2016	Bk 2, p. 20	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	Poly28SR2	ADA916	28S	7/26/2016	Bk 2, p. 20	EI	taq	NEB18S	20
Paramonotus sp.	STB521	5.9	MICOIintF	APN085	COI	10/18/2017	Bk. 5, p. 118	EI	taq	LERAY5	20
Paramonotus sp.	STB521	5.9	jgHCO2198	APN086	COI	10/18/2017	Bk. 5, p. 118	EI	taq	LERAY5	20
Paramonotus sp.	STB525	18.9	600R	APM926	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	TimA	APM924	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	1100R	APM925	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	600F	APM930	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	HNRV	APM931	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	1100F	APM932	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	LSU5	APN936	28S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	LSUD6-3	APN040	28S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB525	18.9	Poly28SF1	APN037	28S	9/26/2017	Bk. 5, p. 68-69	EI	taq	NEB18S	20
Paramonotus sp.	STB525	18.9	Poly28SR2	APN036	28S	9/26/2017	Bk. 5, p. 68-69	EI	taq	NEB18S	20
Paramonotus sp.	STB526	34.9	600R	APM929	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB526	34.9	TimA	APM927	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB526	34.9	1100R	APM928	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20

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Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB526	34.9	600F	APM933	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB526	34.9	HNRV	APM934	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB526	34.9	1100F	APM935	18S	9/26/2017	Bk. 5, p. 68-69	EI	phusion	JS3TIMPF	20
Paramonotus sp.	STB523	11.9	LSU5	ADB064	28S	5/16/2017	Bk 4, p. 35-36	EI	taq	NEB18S	20
Paramonotus sp.	STB523	11.9	LSUD6-3	ADB065	28S	5/16/2017	Bk 4, p. 35-36	EI	taq	NEB18S	20
Paramonotus sp.	STB523	11.9	Poly28SF1	ADB068	28S	5/16/2017	Bk 4, p. 35-36	EI	taq	NEB18S	20
Paramonotus sp.	STB523	11.9	Poly28SR2	ADB072	28S	5/16/2017	Bk 4, p. 35-36	EI	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	600R	ACY011	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	Tim A	ACY008	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	1100R	ACY009	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	600F	ACY013	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	HNRV	ACY014	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	1100F	ACY016	18S	6/6/2016	Bk 2, p. 10	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	LSU5	ACY037	28S	6/9/2016	Bk 2, p. 14	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	LSUD6-3	ACY038	28S	6/9/2016	Bk 2, p. 14	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	Poly28SF1	ACY041	28S	6/9/2016	Bk 2, p. 14	WB	taq	NEB18S	20
Paramonotus sp.	STB531	7.8	Poly28SR2	ACY042	28S	6/9/2016	Bk 2, p. 14	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	600R	ADA929	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	Tim A	ADA927	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	1100R	ADA928	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	600F	ADA933	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	HNRV	ADA934	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB532	3.7	1100F	ADA935	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	600R	ADA932	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	Tim A	ADA930	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	1100R	ADA931	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	600F	ADA933	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	HNRV	ADA937	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	1100F	ADA938	18S	9/21/2016	Bk 2, p. 84	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	LSU5	ADA941	28S	2/3/2017	Bk 3, p. 4	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	LSUD6-3	ADA942	28S	2/3/2017	Bk 3, p. 4	WB	taq	NEB18S	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB533	10	Poly28SF1	ADA947	28S	2/3/2017	Bk 3, p. 4	WB	taq	NEB18S	20
Paramonotus sp.	STB533	10	Poly28SR2	ADA948	28S	2/3/2017	Bk 3, p. 4	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	600R	AHM845	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	Tim A	AHM843	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	1100R	AHM844	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	600F	APM949	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	HNRV	APM951	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	1100F	APM950	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH601	14.9	LSU5	APM875	28S	9/13/2017	Bk. 5, p. 32-33	WB	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH601	14.9	LSUD6-3	APM876	28S	9/13/2017	Bk. 5, p. 32-33	WB	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH601	14.9	Poly28SF1	APM883	28S	9/13/2017	Bk. 5, p. 32-33	WB	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH601	14.9	Poly28SR2	APM884	28S	9/13/2017	Bk. 5, p. 32-33	WB	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH604	39.8	600R	AHM851	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	Tim A	AHM849	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	1100R	AHM850	18S	8/25/2017	Bk. 4, p. 134	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	600F	AHM858	18S	8/28/2017	Bk. 4, p. 141	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	HNRV	AHM859	18S	8/28/2017	Bk. 4, p. 141	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	1100F	AHM860	18S	8/28/2017	Bk. 4, p. 141	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	LSU5	APM877	28S	9/13/2017	Bk. 5, p. 32-33	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	LSUD6-3	APM878	28S	9/13/2017	Bk. 5, p. 32-33	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	Poly28SF1	APM885	28S	9/13/2017	Bk. 5, p. 32-33	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	Poly28SR2	APM886	28S	9/13/2017	Bk. 5, p. 32-33	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH604	39.8	MICOIintF	APN128	COI	12/10/2017	Bk. 6, p. 40	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH604	39.8	jgHCO2198	APN937	COI	12/10/2017	Bk. 6, p. 40	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH605	1.2	600R	APN099	18S	10/19/2017	Bk. 5, p. 127	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH605	1.2	Tim A	APN097	18S	10/19/2017	Bk. 5, p. 127	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH605	1.2	1100R	APN098	18S	10/19/2017	Bk. 5, p. 127	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH605	1.2	600F	APM940	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH605	1.2	HNRV	APM942	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH605	1.2	1100F	APM941	18S	12/10/2017	Bk. 6, p. 40	WB	taq	NEB18S	20
Proschizorhynchella sp.	PSH606	1.8	600R	AHM863	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Proschizorhynchella sp.	PSH606	1.8	TimA	AHM861	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH606	1.8	1100R	AHM862	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH606	1.8	600F	APM844	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH606	1.8	HNRV	APM845	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH606	1.8	1100F	APM846	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH606	1.8	LSU5	APM879	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH606	1.8	Poly28SF1	APM887	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH606	1.8	Poly28SR2	APM888	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH606	1.8	LSUD6-3	APM880	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH607	3	600R	APM843	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	TimA	APM841	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	1100R	APM842	18S	9/7/2017	Bk. 5, p. 12	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	600F	APM891	18S	9/13/2017	Bk. 5, p. 32-33	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	HNRV	APM892	18S	9/13/2017	Bk. 5, p. 32-33	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	1100F	APM893	18S	9/13/2017	Bk. 5, p. 32-33	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH607	3	LSU5	APM881	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH607	3	Poly28SF1	APM889	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH607	3	Poly28SR2	APM890	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH607	3	LSUD6-3	APM882	28S	9/13/2017	Bk. 5, p. 32-33	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	MICOIintF	APM938	COI	12/10/2017	Bk. 6, p. 40	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH608	36.6	jgHCO2198	APM939	COI	12/10/2017	Bk. 6, p. 40	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH608	36.6	TimA	APN100	18S	10/19/2017	Bk. 5, p. 127	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	1100R	APN101	18S	10/19/2017	Bk. 5, p. 127	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	600F	APN109	18S	10/19/2017	Bk. 5, p. 127	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	HNRV	APN105	18S	10/19/2017	Bk. 5, p. 127	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	600R	APN102	18S	10/19/2017	Bk. 5, p. 127	EI	taq	NEB18S	20
Proschizorhynchella sp.	PSH608	36.6	1100F	APM944	18S	12/10/2017	Bk. 6, p. 40	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH608	36.6	LSU5	APN114	28S	10/25/2017	Bk. 5, p. 140	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH608	36.6	Poly28SF1	APN118	28S	10/25/2017	Bk. 5, p. 140	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH608	36.6	Poly28SR2	APN119	28S	10/25/2017	Bk. 5, p. 140	EI	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH608	36.6	LSUD6-3	APN115	28S	10/25/2017	Bk. 5, p. 140	EI	phusion	JS3TIMPF	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Proschizorhynchella sp.	PSH706	18.9	MICOlintF	APM962	COI	1/20/2017	Bk. 6, p. 82	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	18.9	jgHCO2198	APM963	COI	1/20/2017	Bk. 6, p. 82	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	2.3	MICOlintF	APM964	COI	1/20/2017	Bk. 6, p. 82	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	2.3	jgHCO2198	APM965	COI	1/20/2017	Bk. 6, p. 82	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH606	1.8	MICOlintF	APM960	COI	1/20/2017	Bk. 6, p. 82	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH606	1.8	jgHCO2198	APM961	COI	1/20/2017	Bk. 6, p. 82	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH607	3	MICOlintF	APM958	COI	1/20/2017	Bk. 6, p. 82	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH607	3	jgHCO2198	APM959	COI	1/20/2017	Bk. 6, p. 82	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	18.9	600R	APN041	18S	9/30/2017	Bk. 5, p. 83	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	TimA	APN043	18S	9/30/2017	Bk. 5, p. 83	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	1100R	APN042	18S	9/30/2017	Bk. 5, p. 83	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	600F	APN052	18S	9/30/2017	Bk. 5, p. 83	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	HNRV	APN053	18S	9/30/2017	Bk. 5, p. 83	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	1100F	APM968	18S	1/29/2018	Bk. 6, p. 83	FL	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH706	19.3	MICOlintF	APM977	COI	1/29/2018	Bk. 6, p. 83	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	19.3	jgHCO2198	APM979	COI	1/29/2018	Bk. 6, p. 83	FL	taq	LERAY5	20
Proschizorhynchella sp.	PSH706	18.9	LSU5	APM984	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	Poly28SF1	APM990	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	Poly28SR2	APM991	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	18.9	LSUD6-3	APM985	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Paramonotus sp.	STB527	3	FOB-Rv	ATW670	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB527	3	ITSFwd	ATW668	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB527	3	ITS-LZ	ATW669	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB527	3	ITS-BJFwd	ATW002	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB527	3	STB5.8SFw	ATW004	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB527	3	ITS2.2	ATW003	ITS	4/8/2018	Bk. 8, p. 7	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB505	4.2	MICOlintF	APM998	COI	2/4/2018	Bk. 6, p. 146	NTB	taq	LERAY5	20
Paramonotus sp.	STB505	4.2	jgHCO2198	APM999	COI	2/4/2018	Bk. 6, p. 146	NTB	taq	LERAY5	20
Paramonotus sp.	STB510	2.2	MICOlintF	APN000	COI	2/4/2018	Bk. 6, p. 146	NTB	taq	LERAY5	20
Paramonotus sp.	STB510	2.2	jgHCO2198	APN001	COI	2/4/2018	Bk. 6, p. 146	NTB	taq	LERAY5	20
Paramonotus sp.	STB521	5.9	ITSFwd	ATW625	ITS	3/29/2018	Bk. 7, p. 136-138	EI	taq	ITSTAQ7	10

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Paramonotus sp.	STB521	5.9	ITS-LZ	ATW654	ITS	3/29/2018	Bk. 7, p. 136-138	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB521	5.9	ITS-BJFwd	ATW655	ITS	3/29/2018	Bk. 7, p. 136-138	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB521	5.9	ITS2.2	ATW657	ITS	3/29/2018	Bk. 7, p. 136-138	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB521	5.9	STB5.8SRv	ATW656	ITS	3/29/2018	Bk. 7, p. 136-138	EI	taq	ITSTAQ7	10
Paramonotus sp.	STB521	5.9	FOB-Rv	ATW658	ITS	4/7/2018	Bk. 7, p. 149	EI	taq	ITSTAQ7	10
Proschizorhynchella sp.	PSH612	1.8	MICOIintF	APN024	COI	2/13/2018	Bk. 7, p. 35	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH612	1.8	jgHCO2198	APN025	COI	2/13/2018	Bk. 7, p. 35	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH611	1.3	LSU5	APN028	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Proschizorhynchella sp.	PSH611	1.3	Poly28SF1	ATW579	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Proschizorhynchella sp.	PSH611	1.3	Poly28SR2	ATW580	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Proschizorhynchella sp.	PSH611	1.3	LSUD6-3	APN029	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Paramonotus sp.	STB537	3	MICOIintF	AMP994	COI	2/4/2018	Bk. 6, p. 146	WB	taq	LERAY5	20
Paramonotus sp.	STB537	3	jgHCO2198	APM995	COI	2/4/2018	Bk. 6, p. 146	WB	taq	LERAY5	20
Paramonotus sp.	STB606	4.6	1100R	AHM838	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB606	4.6	600R	AHM839	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB606	4.6	TimA	AHM837	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB606	4.6	600F	AHM840	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB606	4.6	1100F	AHM842	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB606	4.6	HNRV	AHM841	18S	7/17/2017	Bk. 4, p. 110	ISP	taq	NEB18S	20
Paramonotus sp.	STB536	6.1	LSU5	APN026	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Paramonotus sp.	STB536	6.1	LSUD6-3	APN027	28S	2/13/2018	Bk. 7, p. 35	WB	taq	18STAQ	20
Paramonotus sp.	STB536	6.1	Poly28SF1	ATW577	28S	2/13/2018	Bk. 7, p. 35	WB	phusion	JS3TIMPF	20
Paramonotus sp.	STB536	6.1	Poly28SR2	ATW618	28S	3/8/2018	Bk. 7, p. 98	WB	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH706	1	LSU5	APM986	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH706	1	LSUD6-3	APM987	28S	2/4/2018	Bk. 6, p. 146	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH709	1	Poly28SF1	ATW621	28S	3/8/2018	Bk. 7, p. 98	FL	phusion	JS3TIMPF	20
Proschizorhynchella sp.	PSH709	1	Poly28SR2	ATW622	28S	3/8/2018	Bk. 7, p. 98	FL	phusion	JS3TIMPF	20
Paramonotus sp.	STB527	3	MICOIintF	ATW623	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20
Paramonotus sp.	STB527	3	jgHCO2198	ATW624	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20
Paramonotus sp.	STB528	2.2	MICOIintF	ATW625	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20
Paramonotus sp.	STB528	2.2	jgHCO2198	ATW626	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Proschizorhynchella sp.	PSH603	2.4	MICOlintF	ATW627	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH603	2.4	jgHCO2198	ATW628	COI	3/13/2018	Bk. 7, p. 115	EI	taq	LERAY5	20
Proschizorhynchella sp.	PSH704	0.2	Poly28SF1	ATW629	28S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH704	0.2	Poly28SR2	ATW630	28S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH705	19.3	TimA	ATW631	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH705	19.3	600R	ATW632	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH705	19.3	600F	ATW636	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH705	19.3	HNRV	ATW638	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH705	19.3	1100F	ATW637	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH705	19.3	1100R	ATW642	18S	3/14/2018	Bk. 7, p. 118	FL	taq	NEB18S	10
Proschizorhynchella sp.	PSH708	2.3	TimA	ATW643	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH708	2.3	1100R	ATW643	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH708	2.3	600F	ATW639	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH708	2.3	HNRV	ATW641	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH708	2.3	1100F	ATW640	18S	3/13/2018	Bk. 7, p. 115	FL	taq	NEB18S	20
Proschizorhynchella sp.	PSH708	2.3	600R	ATW643	18S	3/14/2018	Bk. 7, p. 118	FL	taq	NEB18S	20
Paramonotus sp.	STB536	6.1	MICOlintF	APN022	COI	2/13/2018	Bk. 7, p. 35	WB	taq	LERAY5	20
Paramonotus sp.	STB536	6.1	jgHCO2198	APN023	COI	2/13/2018	Bk. 7, p. 35	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH608	36.6	ITSFwd	ATW036	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH608	36.6	GR-Rv	ATW037	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH608	36.6	DL-Fw	ATW041	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH608	36.6	ITS2.2	ATW043	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH608	36.6	STB5.8SFw	ATW042	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH606	1.8	ITSFwd	ATW033	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH606	1.8	GR-Rv	ATW034	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH606	1.8	DL-Fw	ATW038	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH606	1.8	STB5.8SFw	ATW039	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH606	1.8	ITS2.2	ATW040	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH706	18.9	ITSFwd	ATW026	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH706	18.9	GR-Rv	ATW032	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH706	18.9	DL-Fw	ATW020	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Proschizorhynchella sp.	PSH706	18.9	STB5.8SFw	ATW022	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH706	18.9	ITS2.2	ATW021	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH604	39.8	ITSFwd	ATW029	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH604	39.8	GR-Rv	ATW030	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH604	39.8	DL-Fw	ATW017	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH604	39.8	ITS2.2	ATW018	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH604	39.8	STB5.8SFw	ATW019	ITS	4/15/2018	Bk. 8, p. 27	EI	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH705	19.3	ITSFwd	ATW073	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH705	19.3	GR-Rv	ATW074	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH705	19.3	DL-Fw	ATW075	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH705	19.3	ITS2.2	ATW077	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH705	19.3	STB5.8SFw	ATW076	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH611	1.3	ITSFwd	ATW078	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH611	1.3	GR-Rv	ATW079	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH611	1.3	DL-Fw	ATW080	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH611	1.3	ITS2.2	ATW082	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH611	1.3	STB5.8SFw	ATW081	ITS	4/29/2018	Bk. 8, p. 50	FL	taq	ITSTAQ8	10
Paramonotus sp.	STB511	5.1	FOB-Rv	ATW095	ITS	4/29/2018	Bk. 8, p. 50	ISP	taq	ITSTAQ7	10
Paramonotus sp.	STB511	5.1	ITSFwd	ATW094	ITS	4/29/2018	Bk. 8, p. 50	ISP	taq	ITSTAQ7	10
Paramonotus sp.	STB511	5.1	ITSLZ	ATW096	ITS	4/29/2018	Bk. 8, p. 50	ISP	taq	ITSTAQ7	10
Paramonotus sp.	STB511	5.1	ITS2.2	ATW099	ITS	4/29/2018	Bk. 8, p. 50	ISP	taq	ITSTAQ7	10
Paramonotus sp.	STB511	5.1	STB5.8SFw	ATW098	ITS	4/29/2018	Bk. 8, p. 50	ISP	taq	ITSTAQ7	10
Paramonotus sp.	STB511	5.1	ITSBJFwd	ATW135	ITS	5/8/2018	Bk. 8, p. 71-72	ISP	taq	ITSTAQ7	10
Proschizorhynchella sp.	PSH708	2.3	ITSFwd	ATW110	ITS	5/8/2018	Bk. 8, p. 71-72	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH708	2.3	DL-Fw	ATW075	ITS	5/8/2018	Bk. 8, p. 71-72	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH708	2.3	GR-Rv	ATW111	ITS	5/8/2018	Bk. 8, p. 71-72	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH708	2.3	ITS2.2	ATW114	ITS	5/8/2018	Bk. 8, p. 71-72	FL	taq	ITSTAQ8	10
Proschizorhynchella sp.	PSH708	2.3	STB5.8SFw	ATW113	ITS	5/8/2018	Bk. 8, p. 71-72	FL	taq	ITSTAQ8	10
Paramonotus sp.	STB535	21	MICOIintF	ATW139	COI	5/8/2018	Bk. 8, p. 71-72	WB	taq	LERAY5	20
Paramonotus sp.	STB535	21	jgHCO2198	ATW150	COI	5/8/2018	Bk. 8, p. 71-72	WB	taq	LERAY5	20
Proschizorhynchella sp.	PSH605	1.2	ITSFwd	ATW171	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10

Appendix C (continued)

Putative Species	Specimen	Specimen Conc. (uL)	Primer	Number	Gene	Date sequenced	Notebook	Location	Polymerase	Program	Primer Conc. (uM)
Proschizorhynchella sp.	PSH605	1.2	GR-Rv	ATW172	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH605	1.2	ITS2.2	ATW175	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH605	1.2	DL-Fw	ATW173	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH605	1.2	STB5.8SFw	ATW174	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH609	1.2	GR-Rv	ATW177	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH609	1.2	ITSFwd	ATW176	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH609	1.2	ITS2.2	ATW180	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH609	1.2	DL-Fw	ATW178	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Proschizorhynchella sp.	PSH609	1.2	STB5.8SFw	ATW179	ITS	5/25/2018	Bk. 8, p. 93-94	WB	taq	ITSTAQ9	10
Paramonotus sp.	STB502	5.5	FOB-Rv	ATW160	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB502	5.5	ITSFwd	ATW159	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB502	5.5	ITSLZ	ATW161	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB502	5.5	ITSBJFwd	ATW162	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB502	5.5	ITS2.2	ATW164	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB502	5.5	STB5.8SFw	ATW163	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB607	3.3	FOB-Rv	ATW142	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB607	3.3	ITSFwd	ATW141	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB607	3.3	ITSLZ	ATW143	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB607	3.3	ITSBJFwd	ATW145	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB607	3.3	STB5.8SFw	ATW145	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB607	3.3	ITS2.2	ATW146	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB608	2	FOB-Rv	ATW148	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB608	2	ITSFwd	ATW147	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB608	2	ITSBJFwd	ATW150	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB608	2	ITSLZ	ATW149	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ7	10
Paramonotus sp.	STB608	2	ITS2.2	ATW152	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10
Paramonotus sp.	STB608	2	STB5.8SFw	ATW151	ITS	5/25/2018	Bk. 8, p. 93-94	NTB	taq	ITSTAQ9	10