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ORIGINAL PAPER

Preliminary application of DNA barcoding toward the detection of viable plant propagules at an initial, international point-of-entry in Georgia, USA

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Abstract Over 90% of global commercial trade occurs between seaports, which are initial points-of-entry for nonnative, potentially invasive propagules. As such, there is a need to develop means to both rapidly intercept and identify propagules as they arrive. Here, we focus on plant propagules that are assumed to be non-native, in seed form. Because standard morphological techniques alone are laborious and require taxonomic expertise, we sought to address if identification through barcoding of the plastid DNA (*rbcL* + *matK* genes) of plant seeds could improve current processes in the early detection and rapid response to prevent entry/establishment of nonnative plant species. This research conducted a preliminary foray to evaluate the utility of widely accepted plant plastid DNA barcodes to identify plant propagules (seeds, hereafter) collected from the air-intake grilles of refrigerated shipping containers of a single agricultural commodity arriving at the Port of Savannah, Georgia, USA. We ask four questions: (1) Can DNA barcoding be used to detect seeds collected from shipping containers at the port? (2) What is the genetic composition of propagules entering the port? (3) How do morphological identifications compare to those

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based on genetic analysis? (4) Are nonnative invasive plant species present on shipping containers entering the Port of Savannah? This research collected 11,044 seeds from 628 refrigerated shipping containers between 2015 and 2017. Seeds were then morphologically sorted into *Seed Types*. Barcoding of the *matK* and *rbcL* gene regions of the plastid genomes directly isolated from seeds resulted in poor amplification. This is likely due to a host of potential confounding factors. Therefore, we germinated seeds and utilized leaf-tissues for sequencing of these two gene regions. From BLASTn analyses, results returned top hits for a variety of species, with up to 22 possible nonnative plant species and one definite Federal Noxious Weed. This work investigates the interception application of DNA barcoding to improve agro- and bio-security issues posed by nonnative and invasive species. Though this study required the germination of the seeds to obtain leaf-tissue suitable for our DNA barcoding method, we effectively demonstrated seed viability. Our seed identification process was lengthy and understandably not feasible for real-time application. Therefore, we seek to improve our methods for future applications by testing other approaches that may better complement morphological identification. Next reasonable steps include improved extraction protocols, metabarcoding to generate DNA barcode sequences directly from groups of seeds harvested from shipping containers and implementing other next-generation sequencing techniques.

Keywords Biosecurity · Invasive · *matK* · *rbcL* · Propagule · Seed

Introduction

Increasing globalization of national economies through the trade of commodities and finished products for consumption is one anthropogenic activity that has been shown to directly increase the abundance, diversity, and frequency of nonnative invasive plant species (NNIS, hereafter) due to their inadvertent transport and introduction (Westphal et al. 2008; Hulme 2009; Simberloff et al. 2013). In many cases, the movement and subsequent establishment of NNIS plant propagules have a significant and negative impact on native plant populations (Lonsdale 1999; Lockwood et al. 2005; Colautti et al. 2006; Hietala et al. 2018). The magnitude of this propagule pressure and the subsequent success of NNIS propagules can depend on the size and quantity of individual propagule tissues or individuals and the frequency of introduction events (Lockwood et al. 2005; Catford et al. 2009). The location of introduction also matters because abiotic and biotic factors contribute to the success or failure of propagule establishment, and therefore, nonnative species and invasion success (Catford et al. 2009; Cassey et al. 2018). Only recently has the relative importance of propagule pressure on the economic and ecological benefits of NNIS prevention versus mitigation been considered as part of invasive species management programs and improvement of governmental and/or regulatory interception programs (Colunga-Garcia et al. 2013; Brockerhoff et al. 2014; Cassey et al. 2018).

Seaports serve as a nexus point of global trade between continents for NNIS propagule transport and introduction, in addition to the target trade commodities (Blackburn et al. 2015). As such, seaports experience high quantities of propagule pressure of NNIS and other nonnative, but potentially less impactful species. It has been shown that propagules are often passively transported in high frequency and in large quantities on shipping containers (Epanchin-Niell 2017). The terrestrial habitats associated with seaports, referred to here as 'industrial sites', experience substantial disturbance and activity for the purpose of trade, making them unique research sites to understand NNIS biodiversity and abundance at these points-of-entry. These industrial sites may provide a higher probability of introduction and establishment of nonnative propagules, which may then spread to natural and agricultural environments (Shea and Chesson 2002; MacDougall and Turkington 2005; Bradley et al. 2010; Szymura et al. 2018).

In the USA, the United States Customs and Border Protection (Department of Homeland Security; USCBP, hereafter) and the Animal and Plant Health Inspection Service-Plant Pest Quarantine (US Department of Agriculture; APHIS-PPQ, hereafter) work together with the port authority to intercept, collect, identify, and decontaminate potentially viable NNIS of all taxa. These agencies are currently experiencing reduced personnel and capacity while concurrently experiencing increasing trade volumes (Georgia Ports Authority 2019). Therefore, we identified a need to

find more rapid and/or innovative applications for research to develop and test methods for plant propagule collection/sampling, detection, and identification. Additionally, there is a need to make this information more rapidly transmittable to field personnel. Phytosanitary protocols vary from nation to nation, and options for decontamination of potentially damaging propagules and live organisms are limited (Lucardi 2015, personal correspondence; Jones and Seghetti 2015; Hallman and Loaharanu 2016). Furthermore, the collection of plant propagules (seeds, hereafter) is limited, due mostly to their cryptic nature (i.e., small size and/or lack of identifying structures), and downstream identification is time-consuming and poses challenges for the regulatory agencies tasked with NNIS prevention and interception inspections at seaports and other international points-of-entry (USCBP and USDA, APHIS-PPQ).

Internationally, wealthier nations experience more nonnative plant richness due to increased importation (Hulme 2009). Cargo shipping is often an underestimated, extremely large source of products with an estimated 90% of global trade occurring via ocean ways (Hulme 2009). The Port of Savannah located in the State of Georgia, USA, is owned and operated by the Georgia Ports Authority. This seaport and initial international point-of-entry for shipping containers has averaged 14.6% per annum growth in the volume of containers handled and is the largest single container terminal in North America (GPA 2019). Over 4.35 million TEUs (trade equivalent units, 20-ft container volume standard) were handled in fiscal year 2018 alone (GPA 2019). The number of cryptically hitchhiking nonnative propagules can be high both in number and frequency simply from the international exchange of finished and agricultural commodities based on the number of shipping containers handled annually alone. This highlights an immediate need for the development and application of novel and/or innovative methodology to detect, identify, and quarantine viable NNIS propagules.

Typically, intercepted botanical material is identified using morphological methods. Morphological plant identification techniques are time consuming, require a high level of botanical expertise, and in some cases, are unable to delineate species or even genera (Hebert et al. 2003; Hajibabaei et al. 2007; Liu et al. 2017). A technique that may be used for the detection and identification of intercepted seeds from shipping containers is DNA barcoding. This molecular technique, based on sequence variation in short, standardized regions of the plant genome provides a molecular basis to identify specimens through variation and conservation of nucleotides (Hebert et al. 2003; CBOL Plant Working Group 2011; Coissac et al. 2016). For terrestrial plant species, a two-locus universal DNA barcode composed of the *rbcL* + *matK* gene regions of the plant genome has been successful at the documentation and identification of species composition and diversity of local (Kress and Erickson 2007; Burgess et al. 2011) and regional (de Vere et al. 2012; Braukmann et al. 2017) floras. Furthermore, DNA barcoding has the potential to provide rapid and accurate identifications of unknown organisms (Hollingsworth 2011; Hollingsworth et al. 2011), which can be very helpful in cases where identification based on morphology may be particularly challenging due to cryptic and/or ephemeral characteristics (Kress et al. 2015; Vassou et al. 2015), such as root-tissue (Kesanakurti et al. 2011; Jiao et al. 2018; Meiklejohn et al. 2018), leaf fragments (Kress et al. 2005; MacIvor 2016), and seeds (Fang et al. 2016; Ghorbani et al. 2017). Given its ability to identify plant species with small amounts of plant tissue, the process of DNA barcoding may be an ideal tool for identification and improved interception of nonnative propagules at seaports prior to long-distance overland distribution post-establishment (Blackburn et al. 2015). Yet, to our knowledge, the efficacy of the use of DNA barcoding in this context has rarely been empirically tested in this field (Kress 2017).

The overarching goal of this research was to test the potential efficacy and accuracy of plant DNA barcoding to identify plant propagules for potential application by governmental, regulatory agencies in the USA, and to improve the prevention of nonnative plant species introductions, and subsequent establishment at ports-of-entry, such as seaports. This study focused on testing the relative success or failure of DNA barcoding of two plastid regions of plants from seeds and from subsequently germinated seedlings from the Port of Savannah. We utilized the combination of the *rbcL* + *matK* gene regions for this preliminary screening and testing of potential specificity of identification, relative success in amplification, and potential application for large-scale deployment on plant propagules. This study sought to address the following questions: (1) Can DNA barcoding be used

to detect plant propagules (seeds) collected from shipping containers at the port?, (2) What is the genetic composition of propagules entering the port?, (3) How do morphological identifications compare to those based on genetic analysis?, and (4) Are nonnative invasive plant species present on shipping containers entering the Port of Savannah? The climate at the Port of Savannah (Georgia, USA) is subtropical (Long et al. 2007) and the port is frequently visited by agricultural shipping containers originating from tropical regions. These climatic conditions may be an optimal habitat for new taxa to establish in the already disturbed substrate available on the port terminal. Recent floristic surveys of the Port of Savannah indicate that > 30% of the plant species present on-site are nonnative species (Lucardi et al. *in review*), which underscores the importance of seaports being potential points-of-entry for nonnative, and potentially invasive plant species. From this research, we then explore future work for practical utilization of DNA barcoding for the detection, identification, and interception of nonnative plant propagules entering seaports or other international points-of-entry.

Methods

Study site

This study was conducted at the Port of Savannah, Garden City Terminal (GCT, hereafter), located near Savannah, Georgia, USA (32°07′42.5″N 81°09′05.4″W). The GCT is the container terminal for the Port of Savannah and spans 485.6 hectares (1200 acres). The GCT provides the most novel infrastructure and capacity for refrigerated shipping containers on the Atlantic Coast of the USA, with the ability to accommodate 2016 individual containers (GPA 2019). The GPA developed and constructed this innovative refrigerated shipping container storage system, which stacks refrigerated containers associated with personnel-accessible, multi-story racks, equipped with the electrical power to provide the needed power to maintain requisite temperatures for imported commodities within refrigerated shipping containers (GPA 2019). We chose to sample refrigerated shipping containers because they possess air intake grilles, metal grates, onto which debris and propagules can accumulate during their shipment history.

Sampling

The utilization of DNA barcoding to identify plant propagules, introduced into the country via shipping containers, targeted a single agricultural commodity known to originate from a single port of origin in South America that was then trans-shipped (GPA 2019, personal correspondence). *Trans-shipping* is a means of reducing congestion at and around the Isthmus of Panama, where canal expansion and increased crossing costs make trans-shipping a cost and time efficient means of frequent and high-volume commodity transport. In this case, *trans-shipping* means that shipping containers, refrigerated or ambient (dry-boxes), are transported by a ship operating in the Pacific Basin then temporarily stored and plugged in at the Panama Canal container terminal, where containers are stored for a period of time, weather permitting (generally 24 to 72 h; GPA personal correspondence). A separate ship, which operates in the Atlantic and Gulf of Mexico Basin(s), then picks up these *trans-shipped* containers and finally arrives at the Garden City Terminal at the Port of Savannah; other eastern USA seaports are sometimes visited before or after the target commodity containers reach GCT in Savannah.

Sampling was conducted during two time periods: (1) August 2015 to February 2016 and (2) August 2016 to February 2017. These months are the import season for our target agricultural commodity into the USA from South America. The private entities associated with our data collection, the agricultural commodity, and the importers have been anonymized in our agreement with GPA, and this information does not meaningfully or directly contribute to answering the questions posed in this research. This preliminary research sought 2 years (seasons) of import data for this commodity as a test of conceptual implementation of DNA barcoding.

The GPA, in cooperation with the USCBP, provided access to a subsample of the target-containers, with parameters for sampling based on a combination of arrival projections into GCT and our accessibility to on-site refrigerated containers. Refrigerated shipping containers for our target agricultural commodity are generally on-port for less than 24 h before being

transported off-site. According to GPA, containers could be transported as far as Chicago, Illinois, USA, by semi-truck within 24 to 48 h. During this short window of time when our targeted refrigerated containers were on-terminal, we would arrive at the GCT to sample the air-intake grille surface; we aimed for approximately 2-week intervals during each sampling period (Figure S1). The first sampling season consisted of 33 visits between August 2015 through March 2016, and the second sampling season, strongly influenced by Hurricane Matthew, consisted of fewer visits (14 sampling visits) from August 2016 through February 2017. During each visit, we used a backpack vacuum (110 V), with standard attachments, to access the air-intake grilles. The extension tube associated with the vacuum was fitted with fine, synthetic textile inserts, with an estimated pore size < 0.2 mm, to capture as much of the debris off the air-intake grilles, including our focal research propagules, plant fruits/seeds. After the sampling of each target container, the textile insert was placed into an individual plastic bag with internal labeling designations and a fresh cloth insert was inserted prior to vacuuming the next target container's air-intake grille. All individual plastic bags from each container were then placed in a larger, date-of-sample, plastic bag for downstream processing, including sorting based on rudimentary morphological characteristics.

Initially, large seed mixtures were sorted into morphotypes (i.e., *Seed Types*, hereafter) using stereo microscopes and rudimentary seed botanical taxonomic characteristics. Many *Seed Types* had very few representative fruits/seeds collected, and, in many cases, initial morphological assessments were difficult. Germination trials on samples of seeds allowed us to focus our efforts on identifying the five unique *Seed Types* that germinated in the greatest number. These morphological *Seed Types* were authoritatively identified (by Marsico and Reed) as: Type 1 (*Saccharum spontaneum* L.), Type 7 (originally mis-identified morphologically as *Tragopogon* sp., and subsequently confirmed as *Typha* sp.), Type 11 (*Erechtites hieracifolia* (L.) Raf.), Type 14 (*Arundo donax* L.), and Type 16 (*Andropogon* sp.).

DNA barcode analysis

Preliminary DNA barcoding trials on a subset of collected fruits/seeds were tested. Because the barcoding of seeds is a destructive sampling process, we limited our preliminary assessment to two seeds from each of five *Seed Types*. DNA isolations were performed at the molecular facility at Columbus State University (CSU) in accordance with the protocols outlined in MP Biomedicals™ FastDNA™ SPIN KIT (MP Biomedicals, LLC 3 Hutton Center Drive Suite 100. Santa Ana, CA 92707). Polymerase Chain Reactions (PCRs) were conducted for two plastid loci, *rbcL* and *matK*, using two primers for each gene region: *rbcL*-F and *rbcL*-R; *matK*-xf and *matK*-MALP (Table S1 and S2). The primer sequences used and the required ingredients and temperature profiles for PCR can be found in Supplementary Table S1 and Table S2, respectively. Crude PCR products were sent to Functional Biosciences, Inc. (Functional Biosciences, Inc. 505 South Rosa Road, Suite 238, Madison, WI 53719) for sequencing using the same primers amplified during PCR reactions.

We were unable to generate high-quality, bidirectional DNA barcodes directly from the seeds so we subsequently germinated seeds for each *Seed Type* to generate enough high-quality plant material for DNA extraction and barcoding analysis to obtain identifications based on our two plastid DNA barcode regions. Seeds were germinated, grouped by *Seed Type,* at the USDA Forest Service, Southern Research Station, Forestry Sciences Laboratory (Athens, Georgia). Germination conditions were initially provided in petri dishes with distilled water-moistened filter paper. Dishes were checked and monitored daily for bacterial growth for a term of 14 days, after which seedlings were transferred to soil substrate (Sunshine Mix 1, Sun Gro, Sun Gro Horticulture, 770 Silver Street, Agawam, MA 01001) and into 4-in plastic pots for further growth. When seedlings produced more than the first set of true seed-leaves, approximately 1-cm^2 of leaf-tissue was sampled, stored in a #1 size coin envelope, and placed in silica gel, with indicator, to dry. DNA isolations, PCR, and sequencing protocols followed those outlined for seeds stated above (Table S1 and S2).

Sequencing assembly and bioinformatic analysis

Sequence editing and base calling were conducted in *Geneious* 11.1.3 (Kearse et al. 2012). Forward and reverse sequences were assembled into contiguous DNA barcodes using De Novo assembly. Each

individual DNA barcode for both sequenced loci was queried to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using BLASTn (Basic Local Alignment Search Tool) analysis. All queries were assessed for 100% identical matches to GenBank with the highest bit score (a combination of sequence length and percentage identity). The resulting identifications with the highest bit score and 100% match were selected to represent the identification of each *Seed Type* from our sample collections. In cases where multiple species returned a 100% match to the query, all species were listed as possible molecular identifications of the queried sequence. The presence and quality of sequences available on GenBank certainly varies, so we ensured there were representative sequences for each proposed morphological identification. In cases where the seeds were identified to genus, BLAST results returned many species, often within that genus.

Results

Sample recovery

Over the course of two sampling seasons, we collected a total of 11,044 seeds from the air-intake grilles of 628 refrigerated shipping containers of our targeted agricultural commodity entering GCT at the Port of Savannah. The first sampling season, August 2015–February 2016 (Year 1), 5537 seeds were collected, 11 types/seed groups determined, and 331 containers sampled over 15 sampling dates. The second season, August 2016–February 2017 (Year 2), 5507 seeds were collected, 22 types/seed groups determined from 297 containers over 14 sampling dates. In all cases, we were unable to generate high-quality, bidirectional DNA barcodes directly from the seeds, possibly due to low DNA yields or PCR inhibitors to amplification and/or sequencing. We controlled for and were able to exclude contamination due to PCR or isolation processes due to the use of blanks in our extractions. The sequences received from singular seeds were very messy, and when queried to GenBank using BLASTn resulted in *Musa* sp., potentially a contaminant from the environment where the containers were housed. Due to the failed sequencing of the seeds, we germinated seeds for each *Seed Type*. In total, we were able to successfully germinate five *Seed Types* (represented in unequal sample sizes by a total 90 seedlings), on which we conducted DNA barcoding: Types 1, 7, 11, 14, and 16.

Sequence recovery

In total, 90 seedlings across five *Seed Types* were sequenced for our two selected plastid gene regions, *rbcL* and *matK*. A total of 157 barcodes were generated from a possible total of 180 sequences (87% sequence recovery), with the overall percentage sequencing success for *rbcL* (93%), higher than that for *matK* (81%). Among the different *Seed Types*, Type 16 had the greatest sequencing success (94%), with 16 specimens generating 30 barcodes, whereas Type 11 had the lowest (67%; 9 specimens generating 12 barcodes) (Fig. 1). Intermediate values were found for Type 1 (92%; 26 specimens generating 48 barcodes), Type 7 (80%; 18 specimens generating 29 barcodes), and Type 14 (90%; 21 specimens generating 38 barcodes) (Fig. 1). Among *Seed Types*, the sequence recovery rate varied between the two loci (Fig. 1). Types 1 and 16 resulted in the highest sequencing success rate for *rbcL* (100%), but rates for *matK* for each of these types were lower, 85% and 88%, respectively (Fig. 1). Type 11 had the lowest sequencing success rate for both *rbcL* (78%) and *matK* (56%), and intermediate values for both gene regions were found for the *Seed Types* 14 and 16 (Fig. 1).

Genetic identification

Overall, DNA barcode sequencing of the 90 seedlings yielded 18 unique genetic haplotypes (Fig. 2; Fig. S2, Table S3). The number of unique haplotypes recovered for the barcode *matK* (N = 10) was higher than that for the barcode *rbcL* (N = 8) (Table S3; Fig. 1a, b). The number of haplotypes for each gene region varied among *Seed Types* (Fig. 3). In all cases the number of haplotypes detected for each *Seed Type* was higher for the *matK* gene region than *rbcL*, except for Type 11 (Fig. 3). We found that the Port of Savannah is a potential point-of-entry for NNIS; in total, we detected four haplotypes with sequences returning matches for NNIS; (Table 1). BLAST results from our pilot study at the Port of Savannah returned top hits for a variety of species, with up to 22 possible nonnative plant species and one definite Federal Noxious Weed identified as *Imperata cylindrica* (L.) Raeusch. among the top matches for two haplotypes and 55 nonnative species

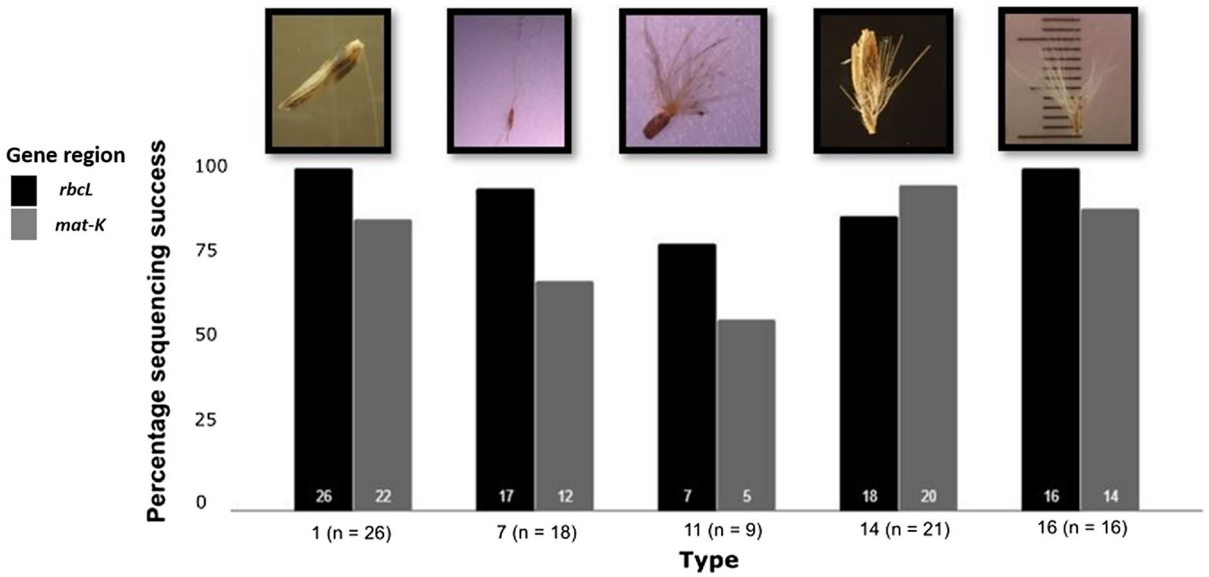

Fig. 1 Sequencing success for two different plastid loci, *rbcL* (black bars) and *matK* (gray bars) sequenced for five different *Seed Types* collected from cargo shipping containers at the Port of Savannah, Georgia, USA. *Seed Type* number and sample size along with a representative picture are included below and above each set of bars, respectively. From left to right, the Seed Types were identified morphologically as *Saccharum spontaneum* L. (1), *Typha* sp. (7), *Erechtites hieracifolius* (L.) Raf. ex DC. (11), *Arundo donax* L. (14), and *Andropogon* sp. (16)

that may be of concern (Table 1) (Federal Noxious Weed List 2019). We also found *Saccharum spontaneum* L., a Federal Noxious Weed, in high abundance with high germination rates entering on refrigerated shipping containers at the Port of Savannah.

Further BLASTn analyses of the sequences on GenBank revealed that the haplotypes detected from GCT at the Port of Savannah spanned three plant families and 19 genera (Table 1). All 26 sequences generated from Type-1 seedlings matched Poaceae (Table 1; Fig. 2). Two *rbcL* haplotypes were found for Type-1 seeds, where 21 sequences equally matched species in the genera *Eulalia, Hemisorghum, Imperata, Lasiorhachis, Miscanthus, Psuedosorghum, Saccharum,* and *Sorghum*; five of the 26 Type-1 barcodes equally matched *Phragmites australis* (Cav.) Trin. ex Steud. and *P. mauritianus* Kunth. (Table 1). For the *mat*K gene region, Type-1 identifications were based on four unique haplotypes, two of which equally matched three *Phragmites* species and another that equally matched three *Saccharum* species; the remaining haplotype equally matched species spanning six genera (Table 1). All the Type-7 *rbcL* and *matK* haplotypes were equally identified to the genus *Typha* (Typhaceae), although two of the *matK* haplotypes were identified to species: *T. latifolia* L. and *T. angustifolia* L. (Table 1; Fig. 2). All Type-11 identifications were identified to family, Asteraceae, based on either *rbcL* or *matK* haplotypes (Table 1; Fig. 2). Of the four Type-11 *rbcL* haplotypes, one was identified to the genus *Conyza*, another to two genera (*Conzya* and *Erigeron)*, with the remaining two haplotypes equally matching species from three genera (*Aster, Conyza, and Erigeron*) (Table 1). Identifications based on the *rbcL* and *matK* gene regions for Type 14 were all identified as species of *Phragmites* (Poaceae), where one *matK* haplotype was identified as *Phragmites australis* L. (Table 1; Fig. 2). All Type 16 haplotypes were identified as Poaceae, where the sole *rbcL* haplotype equally matched species from nine genera and the two *matK* haplotypes matched species within the genus *Andropogon* (Table 1; Fig. 2).

Discussion

Sequence recovery

We aimed to develop a process to rapidly detect potentially nonnative invasive (NNIS) plant

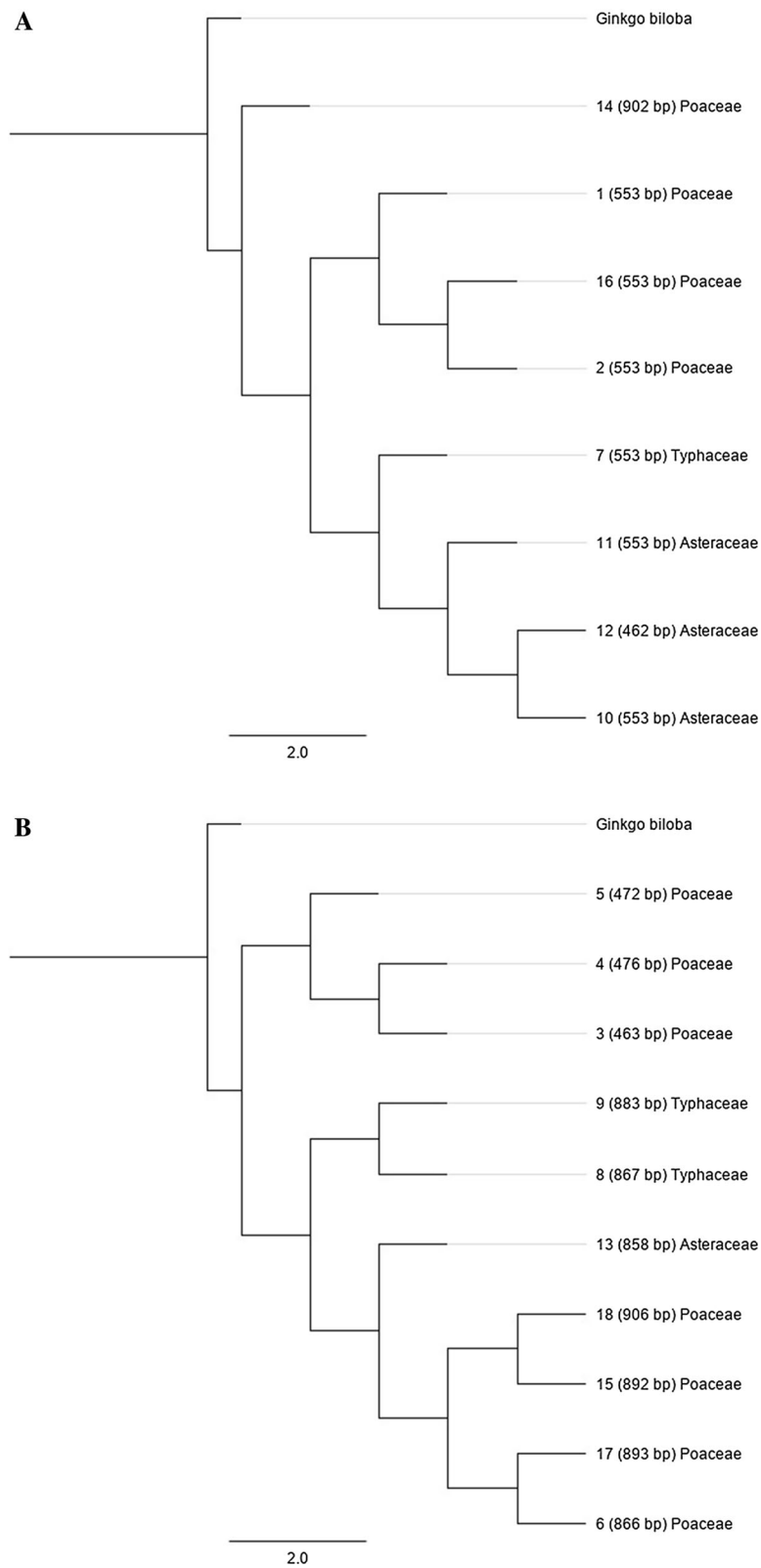

Fig. 2 Cladograms depicting genetic relationships among haplotypes detected for seeds collected from refrigerated shipping containers at the Port of Savannah, Georgia, USA. In total, **a** eight unique haplotypes were found for the *rbcL* gene region and **b** ten unique haplotypes were found for the *matK* gene region. This figure was made using FigTree v1.4.3 with scale bars at the bottom representing the percentage of genetic variation between species with *Ginkgo biloba* L. as the outgroup

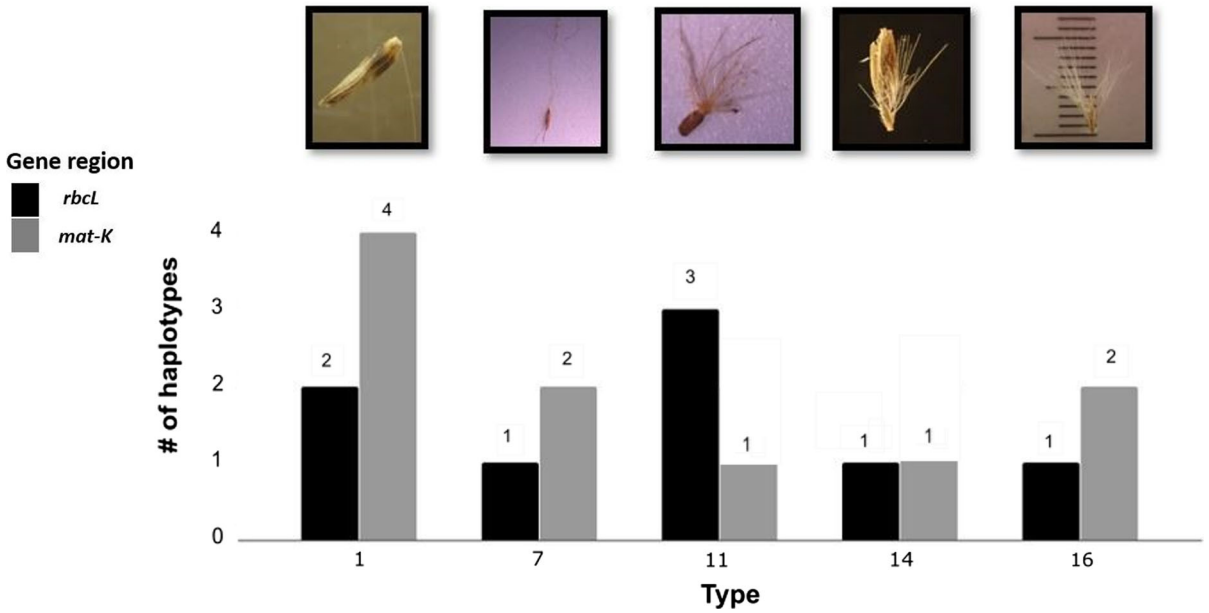

Fig. 3 The number of unique *rbcL* (black bars) and *matK* (gray bars) haplotypes detected from propagules collected on refrigerated shipping containers entering the port of Savannah, Georgia, USA. The number of haplotypes varied among the five *Seed Types* that were identified at the port based on morphology alone. From left to right, the Seed Types were identified morphologically as *Saccharum spontaneum* L. (1), *Typha* sp. (7), *Erechtites hieracifolius* (L.) Raf. ex DC. (11), *Arundo donax* L. (14), and *Andropogon* sp. (16)

propagules (seeds) entering the Port of Savannah on refrigerated shipping containers' air-intake grilles. Our grand goal was to directly obtain DNA barcodes from seeds collected from the GCT on the Port of Savannah. However, our preliminary attempt for the DNA barcoding of seeds indicated that substantial methodological rationale needed to be addressed. Regardless, the aim was also to provide identifications of seeds collected during their hitchhike to the agencies and cooperators. Our effort to conduct DNA barcoding (of regions *rbcL* and *matK*) directly from seeds resulted in low or unsuccessful yields of high-quality DNA. The majority of collected seeds were small and inconspicuous, likely resulting in their ability to be windborne and affixed to air-intake grilles on the refrigerated shipping containers. In the few cases where we extracted detectable DNA, we found that contamination/inhibitors contributed to PCR and/or sequencing failure. We posit that contamination was likely due to chaff, fungal symbionts, chemical contaminants, or other debris (biological and otherwise) attached to the seeds prior to isolation. Although successful DNA barcoding of seeds has been accomplished in other studies, most focused on a particular species (Shahzadi et al. 2010; Gizmondi et al. 2012) or genus (Fang et al. 2016), and few standardized protocols exist across a broad range of plant taxa (Rogers and Bendich 1988; Cristina et al. 2017). Obtaining high-quality DNA as well as resulting amplified PCR products and sequences from seeds appears to be a widespread challenge (Meng and Feldman 2010; Demeke et al. 2012; Li and Trick 2018). Given that seeds collected from shipping containers at a seaport are most likely to be of unknown species composition, it seems reasonable that before a broad scale DNA barcoding pipeline for the genetic identification of seeds can be implemented, further and more extensive, empirical trials on DNA barcoding of seeds across a broad range of plant taxa is a reasonable next step.

Our approach was able to successfully germinate and recover DNA barcode sequences from 90 seeds collected at the seaport from leaf-tissue of successfully germinated seedlings. Our sequence recovery rates were higher, in general, for the *rbcL* gene region (93%) than that for *matK* (81%), comparable to a broad range of other studies (Kress et al. 2010; Burgess et al. 2011; Chen et al. 2015; Braukmann et al.

Table 1 Genetic identification of 18 haplotypes detected from seed propagules collected from refrigerated shipping containers intercepted at the port of Savannah, Georgia

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
1	Poaceae: *Saccharum spontaneum*	*rbcL*-1 (553)	Poaceae	*Phragmites australis* (Cav.) Trin. ex Steud	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-1 (553)	Poaceae	*Phragmites mauritianus* Kunth	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Eulalia siamensis* Bor**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Eulalia quadrinervis* (Hack.) Kuntze**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Hemisorghum mekongense* (A.Camus) C.E.Hubb**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Imperata cylindrica* (L.)**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Lasiorachis hildebrandtii* (Hack.) Stapf**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Miscanthus capensis* (Nees) Andersson**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus floridulus* (Labill.) Warb. ex K.Schum. & Lauterb	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Miscanthus junceus* (Stapf) Pilg**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus sacchariflorus* (Maxim.) Hack	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus sinensis subsp. condensatus* (Hack.) T. Koyama	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus sinensis var. purpurascens* (Andersson) Matsum	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus sinensis subsp. sinensis*	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Miscanthus transmorrisonensis* Hayata	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Psuedosorghum fasciculare* (Roxb.) A.Camus**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	***Saccharum hildebrandtii* (Hack.) Clayton**	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Saccharum* hybrid cultivar	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Saccharum spontaneum* L.	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum arundinaceum* (Desv.) Stapf	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum bicolor* (L.) Moench	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum drummondii* (Nees ex Steud.) Millsp. & Chase	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum halepense* (L.) Pers	100	1022

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum propinquum* (Kunth) Hitchc	100	1022
1	Poaceae: *Saccharum spontaneum*	*rbcL*-2 (553)	Poaceae	*Sorghum timorense* (Kunth) Buse	100	1022
1	Poaceae: *Saccharum spontaneum*	*matK*-3 (476)	Poaceae	*Phragmites australis* (Cav.) Trin. ex Staustra	100	880
1	Poaceae: *Saccharum spontaneum*	*matK*-3 (476)	Poaceae	*Phragmites japonicus* Steud	100	880
1	Poaceae: *Saccharum spontaneum*	*matK*-3 (476)	Poaceae	*P. mauritianus* Kunth	100	880
1	Poaceae: *Saccharum spontaneum*	*matK*-4 (463)	Poaceae	***Phragmites japonicus* Steud**	100	856
1	Poaceae: *Saccharum spontaneum*	*matK*-4 (463)	Poaceae	*Phragmites australis* (Cav.) Trin. Ex Steud.	100	856
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Hemisorghum mekongense* (A.Camus) C.E.Hubb**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Imperata cylindrica* L.**,	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Miscanthus capensis* (Nees) Andersson**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Miscanthus floridulus* (Labill.) Warb. ex K.Schum. & Lauterb	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Miscanthus giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Miscanthus junceus* (Stapf) Pilg**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Miscanthus sacchariflorus* (Maxim.) Hack	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Miscanthus sinensis* Andersson	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Miscanthus transmorrisonensis* Hayata	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Saccharum hildebrandtii* (Hack.) Clayton**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Saccharum* hybrid cultivar	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Saccharum officinarum* L.	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Saccharum perrieri* (A.Camus) Clayton**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Saccharum robustum* E.W.Brandes & Jeswiet ex Grassl**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Saccharum sinense* Roxb**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Saccharum spontaneum* L.	100	872

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Sorghum arundinaceum* (Desv.) Stapf	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Sorghum bicolor* (L.),	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Sorghum drummondii* (Nees ex Steud.) Millsp. & Chase	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	*Sorghum halepense* (L.) Pers	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Sorghum propinquum* (Kunth) Hitchc**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Sorghum timorense* (Kunth) Buse**	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-5 (472)	Poaceae	***Psuedosorghum fasciculare*** (Roxb.) A.Camus	100	872
1	Poaceae: *Saccharum spontaneum*	*matK*-6 (866)	Poaceae	*Saccharum* hybrid cultivar	100	1600
1	Poaceae: *Saccharum spontaneum*	*matK*-6 (866)	Poaceae	*Saccharum officinarum* L.	100	1600
1	Poaceae: *Saccharum spontaneum*	*matK*-6 (866)	Poaceae	*Saccharum spontaneum* L.	100	1600
7	Typhaceae: *Typha* sp.	*rbcL*-7 (553)	Typhaceae	*Typha angustata* Bory & Chaub	100	1022
7	Typhaceae: *Typha* sp.	*rbcL*-7 (553)	Typhaceae	*Typha domingensis* Pers	100	1022
7	Typhaceae: *Typha* sp.	*rbcL*-7 (553)	Typhaceae	*Typha latifolia* L.	100	1022
7	Typhaceae: *Typha* sp.	*rbcL*-7 (553)	Typhaceae	*Typha orientalis* C. Presl	100	1022
7	Typhaceae: *Typha* sp.	*matK*-8 (867)	Typhaceae	*Typha latifolia* L.	100	1022
7	Typhaceae: *Typha* sp.	*matK*-9 (883)	Typhaceae	*Typha angustifolia* L.	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-10 (513)	Asteraceae	*Conyza bonariensis* L. Cronquist	100	948
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-10 (513)	Asteraceae	*Conyza. sumatrensis* (S. F. Blake) Pruski & G. Sancho	100	948
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-10 (513)	Asteraceae	*Erigeron* vernus (L.) Torr. & A.Gray	100	948
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-10 (553)	Asteraceae	*Conyza bonariensis* L. Cronquist	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-10 (553)	Asteraceae	*Conyza. sumatrensis* (S. F. Blake) Pruski & G. Sancho	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	***Aster tongolensis* Franch**	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Conyza canadensis* (L.)	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron anuus* (L.) Desf	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron bellidiastrum* Nutt	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	***Erigeron borealis* (Vierh.) Simmons**	100	1022

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron caespitosus* Nutt	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron canadensis* L.	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron glaucus* Ker Gawl	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron leibergii* Piper	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron poliospermus* A. Gray	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron purpuratus* Greene	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron tenuis* Torr. & A. Gray	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-11 (553)	Asteraceae	*Erigeron yukonensis* Rydb.	100	1022
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	***Aster tongolensis* Franch**	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Conyza canadensis* (L.) Cronquist	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron acris* L.	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron anuus* (L.) Desf	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron aureus* Greene	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron bellidiastrum* Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron caespitosus* Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron canadensis* L.	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron compositus* Pursh	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron denalii* A. Nelson	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron divergens* Torr. & A.Gray	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron elatus* (Hook.) Greene	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron filifolius* (Hook.) Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron flagellaris* A. Gray	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron glacialis* (Nutt.) A.Nelson	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron glaucus* Ker Gawl	100	854

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron grandifloras* Hook	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron humilis* Graham	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron hyperboreus* Hook	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron hyssopifolius* Michx	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron lackschewitzii* G. L. Nesom & W. A. Weber	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron lanatus* Hook	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron leibergii* Piper	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron linearis* (Hook.) Piper	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron lonchophyllus*	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron muirii* A. Gray	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron nivalis* Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron pallens* Cronquist	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron peregrinus* (Banks ex Pursh) Greene	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron poliospermus* A. Gray	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron porsildii* G.L.Nesom & D.F.Murray,Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron pulchellus* Michx	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron pumulis* Nutt	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron purpuratus* Greene	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron speciosus* (Lindl.) DC	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron strigosus* Muhl. ex Willd, Greene	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron uniflorus* L.	100	854
11	Asteraceae: *Erechtites hieracifolius*	*rbcL*-12 (462)	Asteraceae	*Erigeron yukonensis* Rydb	100	854
11	Asteraceae: *Erechtites hieracifolius*	*matK*-13 (858)	Asteraceae	*Conyza bonariensis* (L.) Cronquist	100	1581
11	Asteraceae: *Erechtites hieracifolius*	*matK*-13 (858)	Asteraceae	*Erigeron sumatrensis* Retz.	100	1581

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
14	Poaceae: *Arundo donax*	*rbcL*-14 (553)	Poaceae	*Phragmites australis* (Cav.) Trin. ex Steud	100	1022
14	Poaceae: *Arundo donax*	*rbcL*-14 (553)	Poaceae	***Phragmites mauritianus* Kunth**	100	1022
14	Poaceae: *Arundo donax*	*matK*-15 (902)	Poaceae	*Phragmites australis* (Cav.) Trin. ex Steud	100	1666
14	Poaceae: *Arundo donax*	*matK*-15 (902)	Poaceae	***Phragmites japonicus* Steud**	100	1666
14	Poaceae: *Arundo donax*	*matK*-15 (902)	Poaceae	***Phragmites mauritianus* Kunth**	100	1666
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Anadelphia scyphofera* Clayton**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon abyssinicus* R.Br. ex Fresen**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon aequatoriensis* Hitchc**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon africanus* Franch**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon amethystinus* Steud**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon andringitrensis* (A. Camus) Voronts**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon appendiculatus* Nees**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon ascinodis* C, B, Clarke**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon brazzae* Franch**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon canaliculatus* Schumach**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon distachyos* L.	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon eucomus* Nees**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon floridanus* Scribn	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon gayanus* Kunth**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon glaucescens* Kunth**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon gracilis* Spreng	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon gyrans* Ashe	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon huillensis* Rendle**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon ibityensis* A. Camus**	100	1022

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon insolitus* Sohns**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon laxatus* Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon liebmannii* Hack	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon ligulatus (Stapf) Clayton***	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon mannii* Hook. f., B.R. Arrill. & *I***	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Andropogon mohrii* (Hack.) Hack. ex Vasey	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon schirensis* Hochst**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Andropogon trichozygus* Baker**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Anthaenantia villosa* (Michx.) P.Beauv	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Elymandra subulate* Jacq.-Fél**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Eulalia speciose* (Debeaux) Kuntze**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Exotheca abyssinica* (Hochst. ex A.Rich.) Andersson**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia bracteate* (Humb. & Bonpl. ex Willd.) Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia collina* (Pilg) Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia cymbaria* L. Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia mobukensis* (Chiov.) Chiov**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia newtonii* (Hack.) Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia rudis* Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Hyparrhenia umbrosa* (Hochst.) Andersson ex Clayton**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Monocymbium ceresiiforme* (Nees) Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium claudopus* (Chiov.) Chiov**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium cubense* (Hack.) Nash**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium exile* (Hochst.) Pilg**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium djalonicum* Jacq. -Fél**	100	1022

Table 1 continued

Seed type	Morphological ID	Haplotype (length bp)	Family	Species	Percent Match	Match Score
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium microstachyum* (Desv. ex Ham.) Roseng**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Schizachyrium sanguineum* (Retz.) Alston	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium spicatum* (Spreng.) Herter**	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	*Schizachyrium scoparium* var. *littorale* (Nash) Gould	100	1022
16	Poaceae: *Andropogon* sp.	*rbcL*-16 (553)	Poaceae	***Schizachyrium thollonii* (Franch.) Stapf**	100	1022
16	Poaceae: *Andropogon* sp.	*matK*-17 (893)	Poaceae	*Andropogon gerardii* Vitman	100	1646
16	Poaceae: *Andropogon* sp.	*matK*-17 (893)	Poaceae	*Andropogon mohrii* (Hack.) Hack. ex Vasey	100	1646
16	Poaceae: *Andropogon* sp.	*matK*-18 (890)	Poaceae	*Andropogon floridanus* Scribn	99	1666
16	Poaceae: *Andropogon* sp.	*matK*-18 (890)	Poaceae	*Andropogon glomeratus* (Walter) Britton, Sterns & Poggenb	99	1666
16	Poaceae: *Andropogon* sp.	*matK*-18 (890)	Poaceae	*Andropogon gyrans* Ashe	99	1666
16	Poaceae: *Andropogon* sp.	*matK*-18 (890)	Poaceae	*Andropogon liebmannii* Hick	99	1666

All haplotypes were compared to Genbank using BLASTn analysis. Sequence matches to nonnative invasive species (NNIS) are underlined, Federal Noxious Weeds (FNW) are denoted with an asterisk and nonnative species (NNS) are in bold font

2017). Our level of sequencing success varied across the *Seed Types* that were roughly sorted based on morphology, with one case of *matK* sequencing success rate higher than that for *rbcL* (Fig. 1). This result is not surprising given that numerous studies have shown that sequencing success, particularly that for the *matK* gene region, can be impeded by PCR inhibitors associated with particular groups of taxa, such as the plant families of Asteraceae, Brassicaceae, and Zingiberaceae (Fazekas et al. 2008; Bafeel et al. 2011; Stoeckle et al. 2011). These results demonstrate that DNA barcoding of these two plastid loci results in good sequence recovery rate (87% of the samples we tested yielded high-quality DNA sequences) for germinated plant tissue. Unfortunately, germination of seeds does not allow for rapid identification as we initially had hoped to develop. Still, our research demonstrated an important step toward that effort. We now know from germinating and growing the plants from collected seeds, the morphological identification of the seeds, and DNA barcoding that we have at least one confirmed Federal Noxious Weed (USDA APHIS PPQ) entering the Port of Savannah in proportionately high number (*S. saccharum* L.) as compared to all other *Seed Types*. Also, floristic inventories of green areas in the port reveal roughly one-third of plant species found growing at the Port of Savannah are nonnative species (Lucardi et al. 2020, submitted).

Genetic identification of haplotypes

We also found that the *matK* gene region detected more haplotypes than the *rbcL* gene region, except in the case of Type 11, and this varied across *Seed Types*. Numerous studies have shown that the *matK* gene region, although having lower recovery rates in most studies, typically has higher levels of species resolution than the *rbcL* gene region (CBOL Plant Working Group 2011; Purushothaman et al. 2014; Kang et al. 2017; Poovitha et al. 2017). The *rbcL* gene has been shown to possess slower mutation rates in taxonomically complex groups (i.e., Asteraceae and Poaceae;

Newmaster et al. 2006; Lahaye et al. 2007; Fazekas et al. 2008, 2009; Arolla et al. 2015). This is certainly the case in this study, where species identification was only possible with the *matK* gene region (i.e., Type 7, 14). Type 16 also had better resolution rates based on *matK*, although only to genus (Table 1). In some cases, however, for example with Types 1 and 11, taxonomic resolution was similar between the two gene regions. The *matK* region also failed to resolve to the species level in most cases (i.e., Type 1, 11, and 16), which could be due to the relatively short DNA barcode length ($\sim$ 450 bp) we obtained for some samples (i.e., Poaceae, Type 1 and Asteraceae, Type 11). While this lack of resolution is expected for the more slowly evolving *rbcL* gene region for similar groups of taxa (Elliott and Davies 2014; Li et al. 2014; Su et al. 2016), the short *matK* sequences that we obtained were as informative for distinguishing among these complex and highly diverse taxa.

While we were unsuccessful in generating DNA barcodes directly from seeds, we detected 18 unique genetic haplotypes across the five *Seed Types* derived from leaf tissue that we were able to successfully sequence from germinated seedlings. This result indicated that DNA barcoding can successfully identify multiple plant species, which ultimately indicates that there are a variety of propagules entering the Port of Savannah as passive hitchhikers on the air-intake grilles of refrigerated shipping containers. This result is substantial, regardless of the specific identification of any seed, because genetic diversity at these potential introduction points represents significant and cryptic propagule pressure, mainly due to the high volume and rapidity of the exchange of these containers. Although the number and frequency of invasive species propagules are significant factors associated with propagule pressure (Lonsdale 1999; Lockwood et al. 2005; Colautti et al. 2006; Cassey et al. 2018), Briski et al. (2018) showed that the genetic diversity of propagules may be a more important aspect of invasion success in the invasion process of NNIS (Sakai et al. 2001).

In the USA, particularly impactful NNIS that are plants and have been assessed for risk to the nation's agricultural sustainability are listed and regulated by the Federal Noxious Weed Act (1974); furthermore, some states also have implemented a regulatory list of nonnative plant species. Yet, we also found that the two broadly accepted plastid DNA barcodes (*rbcL* + *matK*) were insufficient to be ready for a broad-scale recommendation/implementation. The inclusion of additional gene regions, in the plastid and nuclear genomes, may result in improved species resolution rates, especially in taxonomically complex and diverse plant families (i.e., Asteraceae, Poaceae) where the *rbcL* and *matK* barcodes fail (Newmaster et al. 2008; Yan et al. 2014; Parveen et al. 2017). The next-generation sequencing technique *metabarcoding*, which has shown the potential to identify mixtures of cryptic specimens like pollen admixtures (Bell et al. 2017) would be a reasonable approach for future research. However, implementation of metabarcoding does come with obstacles including funding, human-capital, and the fact that it is prone to species specific/taxonomic biases which may lead to false negatives for certain species (Coissac et al. 2012; Pawluczyk et al. 2015). We were able to make an interesting foray into utilizing fundamental science in a very applied manner with the potential of meaningful impact to the agencies that are tasked with biosecurity issues in agriculture. Furthermore, we were able to supplement open source databases like GenBank with our sequence information and improve the availability of data for nonnative species for both field personnel and the public.

Morphological and molecular identification of seeds

We found there were three main types of discrepancies between the morphological and genetic identification of propagules recovered from the port. Firstly, seeds that we identified as a single taxon (*Seed Type*) based on morphology were subsequently identified as multiple species that did not match the original morphological designation, although in some cases identifications based on morphology were restricted to higher order taxonomic levels (genus). For example, five Type 1 seeds that were identified as *Saccharum spontaneum* L. morphologically were found to either be *Phragmites australis* or *P. mauritianus* based on DNA barcoding, a trend that was seen in three of the five *Seed Types* (1, 11, 14) screened in this study (Table 1). This result is important because it not only indicates that morphological identifications have the potential to underestimate the number of species coming into ports but also that the identification of seeds based on morphology alone may result in the

misidentification and subsequent inability to detect invasive species propagules entering ports. Secondly, seeds that were identified as a single taxon based on morphology consisted of multiple haplotypes that equally matched the original taxonomic identity and multiple other taxa based on DNA barcoding. For example, 21 of Type 1 seeds were identified as *Saccharum spontaneum* L. (Poaceae) based on morphology but had *rbcL* haplotypes that equally matched species from eight genera, including the taxon designated by morphology (Table 1). In such cases (also see Type 16 *rbcL* haplotypes), identifications based on morphology may be able to inform those from DNA barcoding since the DNA barcode does not seem to be sensitive enough for species-level identification. Thirdly, in two cases, genus-level identifications based on morphology matched those derived from DNA barcoding and additional information from the DNA permitted identification to the species level (see Type 7). In our study, we were only able to achieve species-level designations based on DNA barcoding for twelve individuals (Type 7) based on two *matK* haplotypes (i.e., *Typha latifolia* and *T. angustifolia*). These results suggest that although DNA barcoding may be able to identify some types of seeds on-port, particularly to the family or genus level, morphology is certainly more effective at species-level designations, in our case (Table 1). Collectively, our results highlight that even in the cases where barcoding fails to resolve identification to species, DNA barcoding has the potential to identify cryptic variation and could eventually be included as an addition to morphological methods but should not be considered a replacement for traditional propagule screening methods.

Detection of invasive species at the port

Although our DNA barcoding analysis did not result in the sole identification of NNIS to the species level in most cases, their presence at the port was not ruled out. Based on DNA barcode analysis, 22 different NNIS may be viably entering this seaport, while 55 nonnative species that are not considered invasive (NNS) may also be of concern (Table 1). A Federal Noxious Weed (FNW) and highly impactful NNIS, *Imperata cylindrica* (L.) Raeusch., was among the top matches for two haplotypes (Table 1). Although there is a dearth of comparable studies that use DNA barcoding to identify propagules entering seaports, our results are comparable to numerous studies that utilize taxonomic morphology to show the potential introduction of NNIS propagules at ports (McCullough et al. 2006; Smither-Kopperl 2007; Hulme et al. 2008). Compared to these studies, our results are informative in two ways: (1) DNA Barcoding utilizing the *rbcL* and *matK* gene regions can certainly serve as an alarm bell for future investigations (i.e., there is the potential that NNIS are entering the port), and (2) the time to produce seedlings for DNA barcoding makes it impossible to immediately halt the introduction of NNIS at the point-of-entry. In both cases, our DNA barcoding results represent a substantial contribution to the initiation of more preventative invasive species efforts and combinations of approaches to be utilized by regulatory agencies at ports-of-entry. Collectively, these results indicate that DNA barcoding implementation for NNIS prevention and improvement of existing biosecurity practices has potential to succeed and to reduce the introduction and establishment of NNIS earlier in the invasion process (Richardson et al. 2000; Blackburn et al. 2011). This approach, after more research, can be used to potentially reduce the negative economic impacts and risks to national gross domestic product (GDP) and agricultural and biodiversity conservation interests.

Conclusions

As anthropogenic globalization increases and seaports expand in size and number, biosecurity and the protection of agricultural assets need to become prioritized national and international issues. We hoped that the DNA barcoding of more cryptic, hitchhiking propagules would allow them to be intercepted and identified before being introduced into a naïve region, becoming established, and/or spreading into an invasive species. Through this initial foray into DNA barcoding to improve accuracy of seed collection and plant identification, we found the two plastid-gene regions we utilized were limited in their ability to resolve samples to species consistently. Improved methodology could provide the ability to obtain DNA barcodes from multiple plastid and nuclear regions from a single seed both accurately and precisely. However, our approach to DNA barcoding at the Port of Savannah is one of the first to cooperate with governmental and regulatory agencies, private and

federal industry, and universities in an effort to bring scientific advances to more applied needs, such as the interception and identification of nonnative plant species as compared to morphological identification. For now, DNA barcoding serves as a complement to classical identification; however, with the inclusion of additional barcode regions from the plastid and nuclear genomes, as well as improving DNA extractions from seed, we may see the advent of a new approach that may be applied in the field for the purpose of biosecurity protocols and field personnel to prevent on-going and future NNIS invasion success.

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