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Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs

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Abstract The global transhipment of ballast water and associated flora and fauna by cargo vessels has increased dramatically in recent decades. Invertebrate species are frequently carried in ballast water and sediment, although identification of diapausing eggs can be extremely problematic. Here we test the application of DNA barcoding using mitochondrial cytochrome *c* oxidase subunit I and 16S rDNA to identify species from diapausing eggs collected in ballast sediment of ships. The accuracy of DNA barcoding identification was tested by comparing results from the molecular markers against each other, and by comparing barcoding results to traditional morphological identification of individuals hatched from diapausing eggs. Further, we explored two public genetic databases to determine the broader applicability of DNA barcodes. Of 289 diapausing eggs surveyed, sufficient DNA for barcoding was obtained from 96 individuals (33%). Unsuccessful DNA extractions from 67% of eggs in our study were most likely due to degraded condition of eggs. Of 96 eggs with successful DNA extraction, 61 (64%) were identified to species level, while 36% were identified to possible family/order level. Species level identifications were always consistent between methodologies. DNA barcoding was suitable for a wide range of taxa, including Branchiopoda, Copepoda, Rotifera, Bryozoa and Ascidia. Branchiopoda and Copepoda were respectively the best and worst represented groups in genetic databases. Though genetic databases remain incomplete, DNA barcoding resolved nearly double the number of species identified by traditional taxonomy (19 vs. 10). Notorious invaders are well represented in existing databases, rendering these NIS detectable using molecular methods. DNA barcoding provides a rapid and accurate approach to identification of invertebrate diapausing eggs that otherwise would be very difficult to identify.

Keywords DNA barcodes · Invertebrates · Nonindigenous species · Ship ballast sediment · Species identification · Diapausing eggs

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Introduction

Aquatic nonindigenous species (NIS) are often transported fouled on ships' external surfaces or in ballast water and sediments carried by ships (Carlton

and Geller 1993; Ruiz et al. 2000; Leppäkoski et al. 2002; Bailey et al. 2005; Sylvester and MacIsaac 2010). In order to most effectively utilize limited resources, managers must be able to quantify the risk of NIS introductions associated with different invasion vectors such as ballast water and sediment. One of the best indicators of invasion risk is "propagule pressure", the frequency and density with which NIS are introduced to new habitats (Colautti et al. 2006; Hayes and Barry 2008; Lockwood et al. 2009). An essential but very difficult aspect of measuring propagule pressure is correct identification of the NIS associated with each vector (Bax et al. 2001).

Accurate identification is a principal component of invasion biology, essential for determining that a species is indeed a NIS rather than a locally rare or even endangered species (Bax et al. 2001). Effective management of NIS is generally hindered by insufficient information and resources (Byers et al. 2002; Simberloff et al. 2005; Lodge et al. 2006). Limited systematic, biogeographic and/or historical data often results in an inability to categorize study species as native or nonindigneous (Carlton 2009), while inaccurate or insufficient species identifications could result in misdirected resources against false positives, or worse, inaction against false negatives. As false negatives often lead to late detection of NIS, they can lead to difficulty in eradication and/or stopping further spread, as well as concurrent increases in operational costs (Bax et al. 2001; Simberloff 2009). The success of both prevention and rapid response efforts critically depends on a rapid, accurate and reliable approach to species identifications.

Examination of invertebrate species transported in residual ballast water and sediment of ships has been an active area of recent research (Bailey et al. 2005; Duggan et al. 2005, 2006; Briski et al. 2010). Most of these studies have utilized traditional taxonomic methods to identify individuals collected as active adults or sub-adults, or for diapausing eggs, to identify individuals hatched in the laboratory (Bailey et al. 2003, 2005; Duggan et al. 2006). This approach, however, has some disadvantages. Diapausing eggs will not hatch, even when conditions are favourable, until diapause is broken, and some viable eggs may never hatch in the laboratory as conditions required to induce hatching are complex and vary among taxa (Schwartz and Hebert 1987). Second, traditional taxonomic keys are often effective only for a particular life stage or sex, and juvenile stages, especially nauplii of Copepoda, often cannot be identified. Third, phenotypic plasticity in the character used for species recognition can lead to incorrect identification, and morphologically cryptic species are common in many taxa (Knowlton 1993; Jarman and Elliott 2000). Fourth, traditional taxonomic identification often demands a very high level of expertise and can be very time consuming, with misidentifications or high uncertainty being a common result (Giangrande 2003).

Molecular identification of species through the analysis of a small fragment of the genome represents a more promising approach for species identification, and is already broadly accepted among scientists. DNA identification has been applied to a wide variety of taxa including Copepoda (Bucklin et al. 1999, 2003), Lepidoptera (Brown et al. 1999; Janzen et al. 2005), Culicidae (Shouche and Patole 2000), Araneae (Barrett and Hebert 2005), Scirtothrips (Rugman-Jones et al. 2006), Aves (Hebert et al. 2004), Pisces (Ward et al. 2005; Ivanova et al. 2007) and Mammalia (Hajibabaei et al. 2007; Imaizumi et al. 2007). The approach consists of amplification and sequencing of a specified 'barcode region', followed by comparison of the recovered sequence(s) to available genetic databases to determine species identity (Hebert et al. 2003). The advantages of DNA barcoding are that it allows for identification of species when morphological identification may offer only estimates of higher taxonomic levels or no estimate at all (Darling and Blum 2007), it recognizes cryptogenic species (Bickford et al. 2007; Geller et al. 2010), and it is rapid and cost-effective (Hebert et al. 2003; Wong and Hanner 2008).

However, use of barcodes to identify species is not without drawbacks. The utility of barcodes can be limited by overlap of genetic variation between closely related species (Meyer and Paulay 2005; Monaghan et al. 2005), and by the lack of reference sequences in existing genetic databases (Darling and Blum 2007). The former problem is more challenging as an insufficient 'barcoding gap', which describes the extent of separation between intraspecific variation and interspecific divergence in the selected molecular marker, can prohibit confident species-level identification (Meyer and Paulay 2005). Mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rDNA (16S) have been shown to be broadly applicable for use as DNA barcode regions in animals

because the evolution of these genes is rapid enough to discriminate to the species level (Hebert et al. 2004; Ward et al. 2005; Hajibabaei et al. 2007; Imaizumi et al. 2007; Ivanova et al. 2007) and because of the availability of robust, universal primers (Folmer et al. 1994; Lopez et al. 1997; Zhang and Hewitt 1997). COI is suitable for distinguishing not only closely related species but also phylogeographic groups within species (Gómez et al. 2000, 2007; Hebert et al. 2003).

Here, we use the COI and 16S genes to test the utility of DNA barcodes as a tool for species-level identification of diapausing eggs of aquatic invertebrates found in ships' ballast sediment. For simplicity, the term 'diapausing egg' is used in a broad sense in this paper and includes eggs, statoblasts, and other types of diapausing and non-diapausing dormant (or resting) stages. While the focus of this assessment was diapausing eggs in the strictest sense, we acknowledge that additional dormant stages were not excluded from analysis. The study is based on DNA extraction, PCR amplification using universal COI and 16S primers, and assignment of species identity by comparing resulting sequences with reference databases: GenBank and the Barcode of Life Database (BOLD). We then assess: (1) the accuracy of the DNA barcode identifications by comparing DNA barcode results generated by two molecular markers, and by comparing DNA barcode results to morphological identification; (2) the efficacy of DNA barcoding by comparing the number of species identifications obtained via molecular identification of diapausing eggs versus traditional morphological identification of animals hatched from diapausing eggs; and (3) the utility of DNA barcoding by examining the availability of sequences of invasive invertebrate species in existing reference genetic databases.

Materials and methods

Sample collection and extraction of eggs from sediment

Ballast sediments were collected opportunistically from 13 transoceanic ships arriving to Great Lakes ports (Hamilton, Windsor, Sarnia, Toledo and Detroit) and five transoceanic ships arriving to Sept-Îles, Quebec between June 2007 and September 2008. Approximately 6 kg of sediment was collected from a single tank of each ship. Sediment was homogenized before removal of four 40 g subsamples from each sample. Eggs were separated from sediment using a sugar flotation method (Hairston 1996). Sediment was sieved through a 45 μm sieve, with the retained material washed into centrifuge tubes using a 1:1 mixture (weight:volume) of sucrose and water and centrifuged at approximately 650 rpm (7.7 m s^{-2}) for 5 min. The supernatant was then decanted into a 45 μm mesh sieve and rinsed with water. Diapausing eggs were classified into groups based on size and morphology using a dissecting microscope before DNA extraction and hatching experiments (Fig. 1). Every type of egg was photographed (Fig. 2). A maximum of 15 eggs per group were isolated for DNA extraction. Eggs that appeared completely intact were preferentially selected over those that appeared degraded, when possible; when less than 15 eggs per group were available, all eggs were used regardless of quality (Fig. 2).

Species-level identifications using DNA barcoding

Selected eggs were rinsed thoroughly in double-distilled H_2O several times to remove external debris before DNA extraction. DNA was extracted directly from diapausing eggs using the HotSHOT method (Montero-Pau et al. 2008). Individual diapausing eggs were transferred to 200 μL reaction tubes containing 15 μL of alkaline lysis buffer (NaOH 25 mM, disodium EDTA 0.2 mM, pH 8.0). Once in the buffer, the egg was gently crushed against the side of the tube using a sterile needle under a dissecting microscope. Samples were incubated at 95°C for 30 min and placed on ice for 3 min. Finally, 15 μL of neutralizing buffer (Tris–HCL 40 mM, pH 5.0) was added to each tube. DNA was quantified using a Nanovue spectrophotometer (GE Healthcare UK Limited).

Fragments of the mitochondrial genes COI and 16S were amplified using the universal COI primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2190 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994), and universal 16S primers S1 (5′-CGC CTG TTT ATC AAA AAC AT-3′) and S2 (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Palumbi 1996). PCR

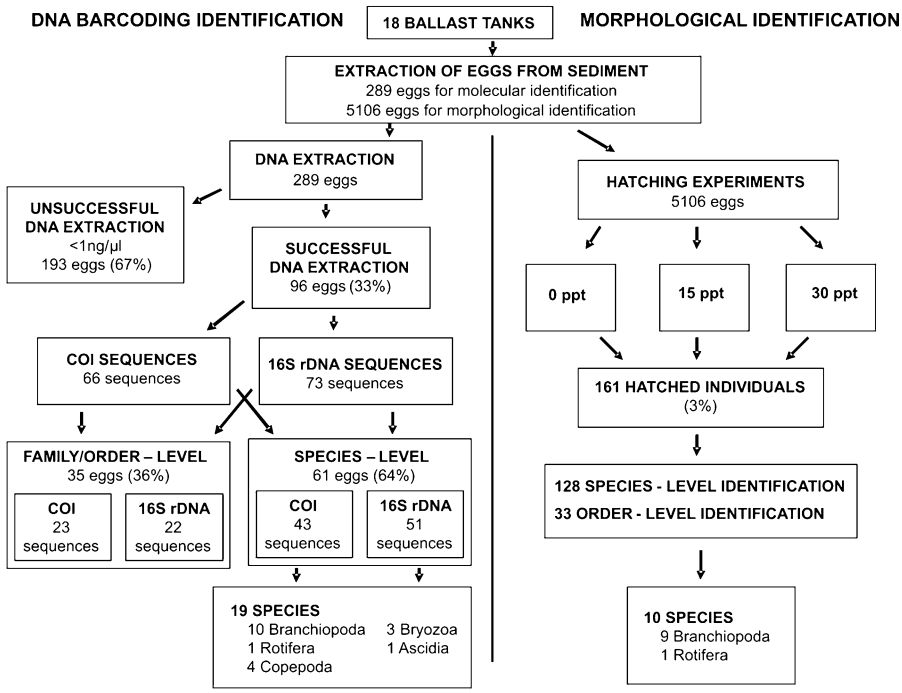

Fig. 1 Schematic representation of DNA barcoding and morphological identification methods used for diapausing egg

reactions were performed in a total volume of 25 µL using 5 µL of DNA extract, 1× PCR buffer, 0.13 mM trehalose, 0.1 µM of each primer, 2.5 mM $MgCl_2$, 0.14 mM dNTPs and 0.4 U Top*Taq* DNA polymerase (Qiagen, Canada). The thermal profile consisted of a 1 min initial cycle at 94°C, followed by 5 cycles of 94°C (40 s), 45°C (40 s) and 72°C (1 min), 35 cycles of 94°C (40 s), 50°C (40 s) and 72°C (1 min), and a final extension of 72°C for 5 min. We did not attempt to concentrate DNA extracts resulting in unsuccessful PCR or to amplify smaller fragments from within the barcode region using primers other than universal Folmer et al. (1994) or Palumbi (1996) primers.

PCR products from eggs were sequenced using an ABI 3130XL automated sequencer (Applied Biosystems, Foster City, CA) and DNA sequences were blasted against GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the nucleotide blast (default parameters). In addition, COI sequences were compared to the BOLD (http://www.barcodinglife.org), using the identification engine BOLD-IDS, with the option 'All Barcode Records on BOLD'. Based on a maximum 4% intraspecific variation in the COI gene reported for Copepoda by Bucklin et al. (2003) and for Cladocera and Amphipoda by Costa et al. (2007), a score resulting in at least 96% similarity to the closest match was deemed a species level identification. As 16S evolves approximately 2 times slower than COI (Adamowicz et al. 2009), 98% similarity was used for discriminations for species level using 16S. Matches lower than 96 and 98% for COI and 16S, respectively, were not assigned to any particular taxonomic level.

Species-level identifications using traditional taxonomy

More than 5,100 diapausing eggs were incubated to conduct traditional morphological identification on hatched individuals (Fig. 1). Sediments were stored in the dark at 4°C for at least 4 weeks to break diapause of dormant eggs before hatching experiments commenced (Grice and Marcus 1981; Schwartz and Hebert 1987; Dahms 1995). Subsequently, diapausing eggs were isolated from 40 g replicate subsamples of sediment using the sugar flotation method described above. Isolated eggs were immediately placed into vials containing 15 mL of sterile synthetic pond water (0 parts per thousand salinity (‰); Hebert and Crease 1980) or sterile seawater medium (15 or 30‰) under a light:dark cycle of 16:8 h at 20°C, using a stratified

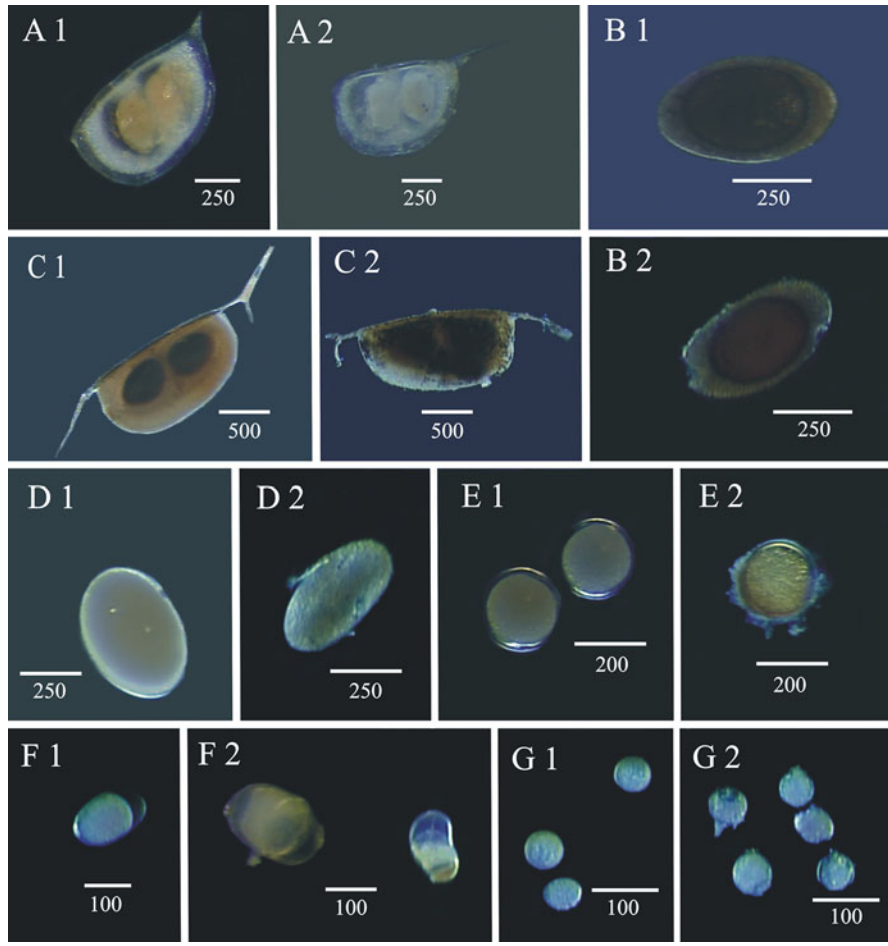

Fig. 2 Resting egg morphotypes with successful (1) and unsuccessful (2) DNA extraction. Branchiopoda: **a1** *Daphnia mendotae*, **a2** *Daphnia* sp., **c1** *Daphnia magna,* **c2** *Daphnia* sp., **d1** *Daphnia magna,* **d2** Branchiopoda, **e1** *Podon intermedius,* **e2** Branchiopoda. Bryozoa: **b1** *Plumatella emarginata*, **b2** *Plumatella* sp. Rotifera: **f1** *Brachionus calyciflorus*, **f2** *Brachionus* spp. Copepoda: **g1** *Leptodiaptomus siciloides*, **g2** various Copepoda. *Scale bars* (μm) are included on each image

random design. The seawater medium was prepared from natural seawater ballast collected from a vessel transiting the Great Lakes, filtered through 2.5 μm Whatman paper filter, and diluted with the sterile, synthetic pond water. Each vial contained between 6 and 81 eggs, depending on the density of eggs in sediment samples.

Three different salinities were used in an attempt to match unknown species to optimum fresh-, brackish- or salt-water habitat to maximize hatching success. Controls containing only hatching media were kept in each treatment group to monitor for the introduction of organisms from the environment. Following Bailey et al. (2005), vials were checked for emergence every 24 h for the first 10 days and every 48 h for a subsequent 10 days, with media renewed every 5 days. Hatched individuals were removed to separate vials and identified morphologically in our laboratory; taxonomic experts were consulted when identifications were uncertain. Hatching percentage was calculated by dividing the total number of animals hatched by the total number of eggs isolated for hatching and multiplied by 100.

Confirmation of species identifications and efficacy of DNA barcoding

A DNA barcoding identification was considered correct when a second methodology gave the same result (i.e. when both molecular markers (COI and

16S), or when morphological identification and one molecular marker, identified eggs from the same group as the same species). To assess efficacy of DNA barcoding we compared the total number of species identified and the variety of taxa identified by DNA barcoding to that of traditional morphological identification of animals hatched from diapausing eggs. Additionally, financial costs and time spent to conduct DNA barcoding *versus* morphological methods were estimated.

GenBank sequence availability

To determine the broader applicability of DNA barcoding for species level identification of diapausing eggs transported by ship sediments, we searched two primary public genetic databases to determine the number of species with available gene sequences in comparison to the number of described species. Rotifera, Bryozoa, Branchiopoda and Copepoda species were investigated for availability of any type of sequence, and specifically for COI and 16S genes, in GenBank (http://www.ncbi.nlm.nih.gov/), and for the COI gene in the BOLD (http://www.barcodinglife.org) on 23 February 2010. In addition, we examined sequence availability of 34 established NIS of Bryozoa, Branchiopoda and Copepoda reported from the Northeast Pacific Ocean (Wonham and Carlton 2005), Laurentian Great Lakes (Ricciardi 2006), and East Coast of Canada (A. Locke, unpublished data) to gain a better understanding of NIS sequence availability on a broader scale. Finally, sequence availability for 55 invasive animal species on the Global Invasive Species Database's "100 of the World's Worst Invasive Alien Species" list (http://www.issg.org/database/welcome) were examined to determine if notorious animal invaders were better represented than NIS in general.

Results

Species-level identifications using DNA barcoding

We isolated 289 diapausing eggs from 18 ballast tanks for DNA barcoding. Of the 289 eggs isolated, DNA was successfully extracted from 96 eggs (33%). Extraction from the remaining 193 eggs resulted in <1 ng/μL of DNA (as quantified using a Nanovue spectrophotometer), and unsuccessful PCR amplification for both COI and 16S DNA fragments. We obtained 139 successful PCR products and 139 sequences using the two sets of universal primers, including 66 COI and 73 16S sequences (Fig. 1). Of the 96 diapausing eggs for which we obtained barcodes, we were able to identify 61 eggs to species level and a further 35 to possible family/order level. Species level identifications were obtained for ten Branchiopoda (44 eggs), one Rotifera (5 eggs), three Bryozoa (6 eggs), four Copepoda (5 eggs) and one Ascidia (1 egg) (Fig. 1; Appendix).

DNA barcoding of diapausing eggs was most successful for species level identification of Branchiopoda belonging to the families Podonidae and Daphniidae. We were able to identify all four Podonidae species and five out of six Daphniidae species. Further, one *Diaphanosoma* was identified to species-level and one to possible genus, while *Moina* and *Bosmina* were poorly represented, resulting in no species identifications. All three Bryozoa species were identified, as was one out of three Rotifera species. Copepoda was the least represented group in the genetic databases; of nine possible species, only four were identified (Appendix).

Six of the 19 species identified by molecular methods are nonindigenous to the Great Lakes region (i.e. *Daphnia magna, Podon intermedius, Pleopis polyphemoides, Cercopagis pengoi, Acartia tonsa* and *Botryllus schlosseri*), while three are nonindigenous to the east coast region (i.e. *D. magna, Calanus euxinus* and *Plumatella emarginata*).

Species-level identifications using traditional taxonomy

Hatching trials were conducted on 5106 diapausing eggs, of which 161 eggs (3%) were successfully hatched. There was no introduction of organisms from the environment into the negative controls. Hatched taxa included Branchiopoda, Copepoda and Rotifera. Morphological species level identification was successful for nine Branchiopoda (109 individuals) and one Rotifera species (19 individuals), but no Copepoda (Appendix). Juvenile naupliar stages of many species of Copepoda are morphologically indistinguishable (Kiesling et al. 2002), thus even taxonomic experts could not identify hatched individuals. Three

of the 10 species identified morphologically were nonindigenous to the Great Lakes region (i.e. *D. magna, P. intermedius* and *P. polyphemoides*), and one was nonindigenous to the east coast region (i.e. *D. magna*).

Confirmation of species identifications and efficacy of DNA barcoding

Of 61 species level identifications by DNA barcoding, 48 were confirmed by a second method: 30 confirmed by both morphological identification and another marker, 14 confirmed only by morphological identification and four confirmed only by another marker (Table 1). Thirteen additional sequences resulted in a species level identification, but were not confirmed by a second method. Forty-four sequences had identification matches between 79 and 95%, resulting in identification only to the possible family/order level (Fig. 1; Appendix). One 16S sequence had an identification match of 99%, but still resulted in only genus level identification as the GenBank reference sequence was only identified to the genus level itself (Appendix). Species level identifications were entirely consistent between methodologies.

Comparison of DNA barcoding and morphological methods revealed that DNA barcoding resolved a greater number of species. While nine Branchiopoda (*D. mendotae, D. parvula, D. magna, D. pulex, D. galeata, Diaphanosoma brachyurum, P. intermedius, P. polyphemoides* and *Evadne normanni*) and one Rotifera (*Brachionus calyciflorus*) were identified by both methods, nine species could be identified only by DNA barcodes: four Copepoda (*Leptodiaptomus siciloides, A. tonsa, Eurytemora affinis* and *C. euxius*), one Branchiopoda (*C. pengoi*), three Bryozoa (*P. emarginata, P. reticulata* and *P. casmiana*) and one Ascidia (*B. schlosseri*) (Fig. 3; Appendix). The success of DNA barcoding identification (19 species) was nearly double that of traditional morphological methods (10 species) (Fig. 1). Further, estimated costs (supplies and labour) and time spent on molecular identification using both markers (unsuccessful tries included) were approximately $1800 (CND) and 72 h, respectively, versus approximately $2600 (CND) and 300 h for morphological identification.

GenBank sequence availability

Our inspection of two public databases revealed the availability of COI and/or 16S sequences for 102, 176, 488 and 416 species for Rotifera, Bryozoa, Branchiopoda and Copepoda, respectively (Table 2, consulted 23 Feb 2010). This represents ~5, 3.5, 54 and 3.5% of described Rotifera, Bryozoa, Branchiopoda and Copepoda species, respectively (Ruppert et al. 2004; BOLD (http://www.barcodinglife.org), consulted 23 Feb 2010) (Table 2). However, searching for COI and 16S sequences of NIS of Bryozoa, Branchiopoda and Copepoda established in the Northeast Pacific Ocean, the Laurentian Great Lakes, and East Coast of Canada resolved available sequences for 7 (44%), 7 (44%) and 2 (100%) species, respectively (Table 3). Of the 55 worst invasive animals reported in the Global Invasive Species

Table 1 List of taxa identified by DNA barcoding using mitochondrial genes COI and 16S

Primary marker	Secondary confirmation	Branchiopoda	Copepoda	Rotifera	Ascidia	Bryozoa	Total
COI	16S and morphological	25	0	5	0	0	30
	Morphological	2	0	0	0	0	2
	16S	3	1	0	0	0	4
	No extra confirmation	2	4	0	1	0	7
16S	Morphological	12	0	0	0	0	12
	No extra confirmation	0	0	0	0	6	6
	Total	44	5	5	1	6	61

Species level identifications were considered accurate if the two genes gave the same results and/or were verified by morphological identification

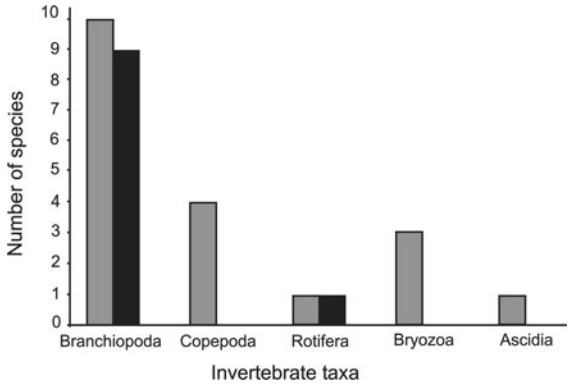

Fig. 3 Number of species identified using DNA barcodes from diapausing eggs (*gray bars*), and morphological identification of hatched animals (*black bar*)

Database, 52 (94%) had COI and/or 16S sequences available.

Discussion

Results from this study indicate that DNA barcoding resolved nearly double the number of species identified by traditional morphological taxonomy (19 vs. 10), and was suitable for a wide range of taxa, including Branchiopoda, Copepoda, Rotifera, Bryozoa and Ascidia. Branchiopoda and Copepoda were respectively the best and worst represented groups in genetic databases. Nevertheless, notorious invaders were well represented, making high priority NIS detectable. Of the 96 diapausing eggs for which we obtained barcodes, we were able to identify 64% to species level and a further 36% to possible family/order level.

Correct identification of species is essential to invasion biology, yet identification of morphologically cryptic species and those which are present as diapausing eggs remains a major challenge. Challenges associated with morphological identification of sub-adult stages render molecular genetic analyses particularly advantageous (Hebert et al. 2003), though accuracy of the method for many taxonomic groups has yet to be demonstrated. For example, Schubart et al. (2008) reported the same COI sequence for two genera of freshwater crabs, while Bucklin et al. (2003) and Costa et al. (2007) reported a 'barcoding gap' for species of Copepoda, Cladocera and Amphipoda. We tested DNA barcoding accuracy for taxa of interest by direct comparison of DNA barcoding results using two gene markers to each other, and by comparison of DNA barcoding and morphological identification results. We found no disagreement among the three methodologies. DNA barcoding using mitochondrial COI and16S genes provides a rapid, accurate method for identification of species from diapausing eggs, and overcomes several problems posed by traditional morphological identification. Even though morphological identification showed the same accuracy as molecular, we estimated that DNA barcoding method is at least 4× times faster and 30% cheaper than morphological

Table 2 Number of described species of Rotifera, Bryozoa, Branchiopoda and Copepoda compared to the number of species for which gene sequences are available in two public databases: GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD (http://www.barcodinglife.org)

Taxa	Number of described species	GenBank			BOLD	Total number of distinct species from GenBank and BOLD (COI + 16S)
		Number of species (all sequences)	Number of species (COI sequence)	Number of species (16S sequence)	Number of species (COI sequence)	
Rotifera	~2,000[a]	205	78	33	34	102
Bryozoa	~5,000[a]	239	76	103	20	176
Branchiopoda	~900[b]	582	364	230	374	488
Copepoda	~12,000[a]	598	256	123	296	416

Comparison was conducted 23 Feb 2010

[a] Ruppert et al. 2004

[b] BOLD (http://www.barcodinglife.org); consulted 23 Feb 2010

Table 3 Gene sequence availability for 34 aquatic NIS recorded in the North Pacific Ocean, the Laurentian Great Lakes and the East Coast of Canada and the 55 worst animal invaders globally in two public databases: GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD (http://www.barcodinglife.org); consulted 23 Feb 2010

Region	Number of NIS	GenBank			BOLD	Total number of distinct species from GenBank and BOLD (COI + 16S)
		Number of species (all sequences)	Number of species (COI sequence)	Number of species (16S sequence)	Number of species (COI sequence)	
Northeast Pacific Ocean[a]	16	7	7	3	6	7
Laurentian Great Lakes[b]	16	7	5	6	6	7
East Coast of Canada[c]	2	2	2	1	2	2
Total	34	16	14	10	14	16
55 worst animal invaders[d]	55	53	46	39	48	52

[a] Wonham and Carlton (2005)

[b] Ricciardi (2006)

[c] Locke (unpublished data)

[d] "100 of the World's Worst Invasive Alien Species" reported on Global Invasive Species Database (http://www.issg.org/database/welcome/)

identification (not including initial equipment costs). Furthermore, notorious invaders such as *C. pengoi* and *B. schlosseri* were identified only by the molecular method. We found DNA barcoding was suitable for a range of taxa, including Branchiopoda, Copepoda, Rotifera, Bryozoa and Ascidia.

By using both markers for each individual egg, and two public databases—GenBank and BOLD—we obtained more sequences and increased the chance of a sequence match, thereby providing higher confidence in identifications. Branchiopoda were the best resolved taxa, possibly due to the fact that about 54% of the species in this group are represented in public genetic databases. In addition, the genus *Daphnia*—which represents 13% of Branchiopoda taxa (Forró et al. 2008)—are used as model organisms for genomics (http://wfleabase.org/) and evolutionary studies (e.g. Hebert et al. 2002). Conversely, only 3.5% of Copepoda have been entered into genetic databases, limiting the current utility of molecular identification methodologies for a group notorious for difficult taxonomic identification (Rombouts et al. 2009). Given morphologically indistinguishable immature stages in this order (Kiesling et al. 2002), augmentation of sequence databases may prove particularly useful for identification of Copepoda in the future.

Unbalanced representation of taxa in sequence databases has been observed by other scientists. Puillandre et al. (2009) reported that sequence availability for marine gastropods is low, with only 4% of taxa identified to species level. Conversely, Wong and Hanner (2008) obtained 99% species identifications for market seafood. Though DNA databases are undergoing continual and rapid expansion, sequence availability for poorly studied taxa remains low compared to commercially important or otherwise better studied taxa. Despite this current limitation, identification of invasive species such as *C. pengoi* and *B. schlosseri* was successful. Sequences exist for 94% of the world's 55 worst invasive animals in the two explored genetic databases. As studies of population genetics of NIS are a major source for sequences, species with small, spatially restricted populations, and those that cause no discernible economical or ecological problems, are rarely studied. Because control and eradication of NIS is usually only possible at the earliest stages of invasion (Bax et al. 2001), DNA barcoding may be especially useful for

management of notorious invaders which are already well represented in genetic databases. Presently, barcoding technology is being used to assay for presence of silver (*Hypophthalmichthys molitrix*) and bighead (*H. nobilis*) carp in waterways adjacent to Chicago, Illinois, as part of an early detection program to prevent spread to Lake Michigan.

Unsuccessful DNA extractions from 67% of eggs in our study were most likely due to the condition of eggs, as eggs that were visibly degraded never hatched. Previous testing of the HotSHOT method (Montero-Pau et al. 2008) for DNA extraction success on freshly cultured *Daphnia pulex* eggs from our lab resulted in 96% success (E. Briski unpublished data) with similar observations reported by Montero-Pau et al. (2008) for diapausing eggs of Rotifera, Cladocera, Anostraca and Notostraca, indicating that our methodology was robust. Considering that diapausing eggs in this study were collected in ships' ballast tanks, degradation of eggs is common and faster than degradation of dormant eggs in natural habitats (E. Briski unpublished data). As universal COI and 16S primers amplify products of similar size, they can be considered a positive control for each other (Ivanova et al. 2007). Failed amplification of one primer can be attributed to primer mismatch. In cases where both markers failed, there is a distinct possibility that the DNA template was degraded (Ivanova et al. 2007). This phenomenon provides possible insight into the viability of diapausing eggs. In our case, 3% of the eggs hatched, while DNA was successfully extracted from 33% of eggs. While the percentage of eggs hatched in the laboratory likely underestimates the true viability of the eggs, the percentage of successful DNA extractions may overestimate viability. The physiology of dormant eggs is very complex, and hatch success depends on the degree of diapause termination, energy content of the eggs, number of non-viable embryos and environmental factors (Carvalho and Wolf 1989; Lavens and Sorgeloos 1996; Gilbert 2004; Pauwels et al. 2007; Briski et al. 2008). As a result, many eggs will not hatch in the laboratory even under favourable conditions (Schwartz and Hebert 1987; Bailey et al. 2003). In contrast, successful DNA extraction may overestimate viability if extraction methods are sensitive enough to amplify degraded DNA of non-viable eggs. Thus, the true viability of diapausing eggs recovered from sediments may be somewhere between the number of hatched individuals and the number of eggs from which DNA was successfully extracted. This information could prove useful to risk assessments, as diapausing eggs that are not viable do not constitute an invasion risk.

Aside from the fact that we found two public sequence databases underpopulated, DNA barcoding still yielded greater taxonomic identification capability than traditional morphological methods. The problem of underpopulated sequence databases is least acute for problematic NIS, which tend to be well represented in existing databases. As sequencing technology improves and DNA barcoding becomes more commonplace, we expect that DNA barcoding for species identifications will become routine for an ever increasing number of taxonomic groups.

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Appendix

See Table 4

Table 4 List of species identified by morphological methods and DNA barcodes

Ship ID	Morphological identification of animals[a]		DNA barcoding identification of eggs[b]						GenBank accession number	
			BOLD		GenBank					
	Organism	#	Organism	COI %	Organism	COI %	Organism	16S %	COI	16S
GL2	*Daphnia mendotae*	3	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475272	GQ343261
			Daphnia mendotae	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475273	GQ343262
			Daphnia mendotae	**99**	*Daphnia mendotae*	**99**	*Daphnia mendotae*	**99**	GQ475274	GQ343263
	Daphnia parvula	6					*Daphnia parvula*	**99**		GQ343264
							Daphnia parvula	**99**		GQ343265
							Daphnia parvula	**99**		GQ343266
							Daphnia parvula	**100**		GQ343267
							Daphnia parvula	**99**		GQ343268
							Daphnia parvula	**100**		GQ343269
GL3	*Brachionus calyciflorus*	7	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**100**	GQ475275	GQ343270
			Brachionus calyciflorus	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**98**	GQ475276	GQ343273
	Diaphanosoma brachyurum	3					*Diaphanosoma brachyurum*	**99**		GQ343272
							Diaphanosoma brachyurum	94		
	Daphnia parvula	6					*Daphnia parvula*	**100**		GQ343271
GL4	*Daphnia magna*	45	*Daphnia magna*	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457315	GQ343274
							Moina sp.	95		
							Moina sp.	95		
							Moina sp.	95		
							Moina sp.	95		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	85		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	85		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	83		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	83		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	84		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	84		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	84		
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	85		

Table 4 continued

Ship ID	Morphological identification of animals[a]		DNA barcoding identification of eggs[b]						GenBank accession number	
			BOLD		GenBank					
	Organism	#	Organism	COI %	Organism	COI %	Organism	16S %	COI	16S
			Moina cf micrura[c]	86	*Moina cf micrura*[c]	86	*Bosmina longirostris*[c]	85		
			Moina cf micrura	86	*Moina cf micrura*	86				
			Moina cf micrura	86	*Moina cf micrura*	86				
			Moina cf micrura	86	*Moina cf micrura*	85				
			Moina cf micrura	86	*Moina cf micrura*	86				
GL5	*Daphnia pulex*	3	*Daphnia pulex*	**99**	*Daphnia pulex*	**99**	*Daphnia pulex*	**99**	GQ475277	GQ343275
GL8	*Podon intermedius*	1	*Podon intermedius*	**99**	*Podon intermedius*	**99**	*Podon intermedius*	**99**	GQ475278	GQ343276
							Plumatella emarginata	**99**		GQ343277
	Calanoida copepod nauplii	6	*Leptodiaptomus siciloides*	**98**	*Leptodiaptomus siciloides*	**98**			GQ466409	
GL10	*Pleopis polyphemoides*	1	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**100**	GQ475279	GQ343278
	Daphnia pulex	9	*Daphnia pulex*	**99**	*Daphnia pulex*	**100**	*Daphnia pulex*	**99**	GQ466410	GQ343279
			Cercopagis pengoi	**100**	*Cercopagis pengoi*	**100**	*Cercopagis pengoi*	**99**	GQ466411	GQ343280
			Cercopagis pengoi	**98**	*Cercopagis pengoi*	**98**	*Cercopagis pengoi*	**99**	GQ466412	GQ343281
							Bosmina fatalis	**86**		
							Bosmina fatalis	**86**		
							Moina cf micrura	**88**		
							Moina cf micrura	**88**		
GL13	*Daphnia magna*	20	*Daphnia magna*	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457316	GQ343282
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457317	GQ343283
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457318	GQ343284
			Daphnia magna	**100**	*Daphnia magna*	**100**	*Daphnia magna*	**98**	GQ457319	GQ343285
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457320	GQ343286
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457321	GQ343287
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457322	GQ343288
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457323	GQ343289
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457324	GQ343290
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**98**	GQ457325	GQ343291
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457326	GQ343292
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457327	GQ343293

Table 4 continued

Ship ID	Morphological identification of animals[a]		DNA barcoding identification of eggs[b]						GenBank accession number	
			BOLD		GenBank					
	Organism	#	Organism	COI %	Organism	COI %	Organism	16S %	COI	16S
			Daphnia magna	**100**	*Daphnia magna*	**99**	*Daphnia magna*	**99**	GQ457328	GQ343294
			Daphnia magna	**100**	*Daphnia magna*	**99**			GQ457329	
			Daphnia magna	**100**	*Daphnia magna*	**99**			GQ457330	
	Brachionus calyciflorus	4	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**98**	GQ466413	GQ466404
							Brachionus rubens	86		
							Brachionus rubens	87		
GL14							*Plumatella reticulata*	**98**		GQ343295
							Plumatella emarginata	**99**		GQ343296
							Plumatella casmiana	**100**		GQ343297
			Botryllus schlosseri	**99**	*Botryllus schlosseri*	**99**			GQ457331	
GL15	*Brachionus calyciflorus*	2	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	*Brachionus calyciflorus*	**99**	GQ466414	GQ466405
	Calanoida copepod nauplii	4	*Eurytemora affinis*[d]	86	*Boeckella meteoris*[d]	84				
GL16			*Diaphanosoma brachyurum*[d]	**100**	*Diaphanosoma* sp.[d]	91			GQ924683	
			Diaphanosoma brachyurum[d]	**100**	*Diaphanosoma* sp.[d]	91			GQ924684	
GL17	*Daphnia galeata*	7	*Daphnia galeata*	**100**	*Daphnia galeata*	**99**	*Daphnia galeata*	**99**	GQ457332	GQ466407
							Daphnia sp.	**99**		GQ343298
	Brachionus calyciflorus	6	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**98**	*Brachionus calyciflorus*	**99**	GQ466415	GQ466406
			Brachionus calyciflorus	91	*Brachionus calyciflorus*	91				
GL21			*Cercopagis pengoi*	**100**	*Cercopagis pengoi*	**99**	*Cercopagis pengoi*	**99**	GQ466416	GQ343299
GL22	Calanoida copepod nauplii	8	*Acartia tonsa*	**99**	*Acartia tonsa*	**100**	*Acartia tonsa*	**98**	GQ466417	GQ466408
			Paraeuchaeta rubra[d]	83	*Boeckella meteoris*[d]	83				
			Boeckella poopoensis[d]	83	*Boeckella meteoris*[d]	83				
EC1	Calanoida copepod nauplii	2	Calanoida[d]	89	*Acartia hudsonica*[d]	79				
			Eurytemora affinis[d]	82	*Acartia tonsa*[d]	81				
							Plumatella emarginata	**98**		GQ343300
							Plumatella emarginata	**98**		GQ343301
EC3	*Daphnia magna*	2	*Daphnia magna*	**98**	*Daphnia magna*	**96**	*Daphnia magna*	**98**	GQ457333	GQ343302
EC12	Calanoida copepod nauplii	6	*Eurytemora affinis*[d]	**99**	*Boeckella poppei*[d]	82			GQ924685	

Table 4 continued

Ship ID	Morphological identification of animals[a]		DNA barcoding identification of eggs[b]						GenBank accession number	
			BOLD		GenBank					
	Organism	#	Organism	COI %	Organism	COI %	Organism	16S %	COI	16S
			Eurytemora affinis[d]	**98**	*Boeckella poppei*[d]	82			GQ924686	
			Eurytemora affinis[d]	89	*Paracalanus parvus*[d]	79				
			Eurytemora affinis[d]	88	*Clausocalanus parapergens*[d]	79				
			Eurytemora affinis[d]	89	*Clausocalanus parapergens*[d]	79				
EC18	Calanoida copepod nauplii	7	*Calanus euxinus*	**99**	*Calanus euxinus*	**98**			GQ457334	
			Austrochiltonia sp.[d]	89	*Acartia tonsa*[d]	81				
EC37	*Evadne normanni*	2					*Evadne normanni*	**99**		GQ343303
							Evadne normanni	**99**		GQ343304
							Evadne normanni	**100**		GQ343305
							Evadne normanni	**100**		GQ343306
	Pleopis polyphemoides	1	*Pleopis polyphemoides*	**99**	*Pleopis polyphemoides*	**99**	*Pleopis polyphemoides*	**100**	GQ475280	GQ343307
			Pleopis polyphemoides	**99**	*Pleopis polyphemoides*	**98**	*Pleopis polyphemoides*	**100**	GQ475281	GQ343308

The maximum identification match (%) of our sequences with those found on BOLD and GenBank is also listed. Match scores ≥96 or ≥98%, for COI and 16S, respectively, were considered species level identifications (highlighted in bold), while <96 or <98%, for COI and 16S, respectively, were considered possible family/order level identifications. GenBank accession numbers for sequences from this study are also provided

= Number of morphologically identified individuals

GL Great Lakes, *EC* east coast of North America

[a] Morphological identification of animals was done from hatched eggs, which were the same distinct group based on size and morphology as those used for DNA barcoding

[b] The same egg is used for both markers (COI and 16S)

[c] Divergence from closest match exceeded 4 or 2% for COI and 16S, respectively, and different markers revealed different closest matches (16S: *Bosmina longirostris* and COI: *Moina cf micrura*)

[d] Divergence from closest match exceeded 4%, and different public database (BOLD and GenBank) revealed different closest matches

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