Molecular Ecology Resources (2009) **9** (Suppl. 1), 181–187 doi: 10.1111/j.1755-0998.2009.02643.x

BARCODING ARTHROPODS

DNA barcoding of marine crustaceans from the Estuary and Gulf of St Lawrence: a regional-scale approach

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Abstract

Marine crustaceans are known as a group with a high level of morphological and ecological diversity but are difficult to identify by traditional approaches and usually require the help of highly trained taxonomists. A faster identification method, DNA barcoding, was found to be an effective tool for species identification in many metazoan groups including some crustaceans. Here we expand the DNA barcode database with a case study involving 80 malacostracan species from the Estuary and Gulf of St Lawrence. DNA sequences for 460 specimens grouped into clusters corresponding to known morphological species in 95% of cases. Genetic distances between species were on average 25 times higher than within species. Intraspecific divergence was high (3.78–13.6%) in specimens belonging to four morphological species, suggesting the occurrence of cryptic species. Moreover, we detected the presence of an invasive amphipod species in the St Lawrence Estuary. This study reconfirms the usefulness of DNA barcoding for the identification of marine crustaceans.

Keywords: Crustacea, DNA barcoding, Gulf of St Lawrence, species diversity

Received 1 October 2008; revision received 30 December 2008; accepted 24 January 2009

Introduction

A biodiversity crisis has emerged in the last decades and we are confronted with the highest extinction rates since the formation of human society (Pimm *et al*. 1995). Mitigation measures are needed but difficulties arise due to the unknown extent of biodiversity and spatial distribution of species assemblages. At the species level, the most investigated of biodiversity levels, it is generally agreed that only a small fraction of all species has been formally described, between 1.5–1.8 million out of an estimated 10 million (Wilson 2003). In the face of dwindling numbers of trained taxonomists, a fast identification method was needed to assist in species inventories. In this context, Hebert *et al*. (2003) proposed the use of a small fragment of mitochondrial DNA from the 5′-end of cytochrome *c* oxidase subunit 1 (COI) gene as a reliable, quick and cost-effective identification system for the whole animal kingdom. Although the method faces strong criticism (Will & Rubinoff 2004; Ebach & Holdrege 2005; Will *et al*. 2005), it was nevertheless found to be effective in a variety of animal groups in both terrestrial and aquatic environments (Hebert *et al*. 2004; Hajibabaei *et al*. 2006; Clare *et al*. 2007; Hubert *et al*. 2008). However, the proposed threshold value of 3% COI sequence divergence for species delineation (Hebert *et al*. 2003) may be problematic in some cases (Barber & Boyce 2006; Burns *et al*. 2007).

Diversity in the sea includes about 300 000 described species, a much smaller number than documented for the terrestrial realm (Gray 1997). However, marine faunal inventories fail to identify about one-third of specimens to the species level (Schander & Willassen 2005) and the existence of cryptic species (Knowlton 1993, 2000; Etter *et al*. 1999) creates another difficulty for biodiversity assessments. Crustaceans are an interesting target for DNA barcoding because they represent one of the most diverse metazoan groups from a morphological and ecological point of view. The subphylum Crustacea includes 52 000 described species divided into 849 families, 48 orders and six classes, but their estimated number is much higher (Martin & Davis 2001). There is no general agreement on crustacean systematics at the higher classification levels (e.g. class) (Boxshall 2007), and recently,

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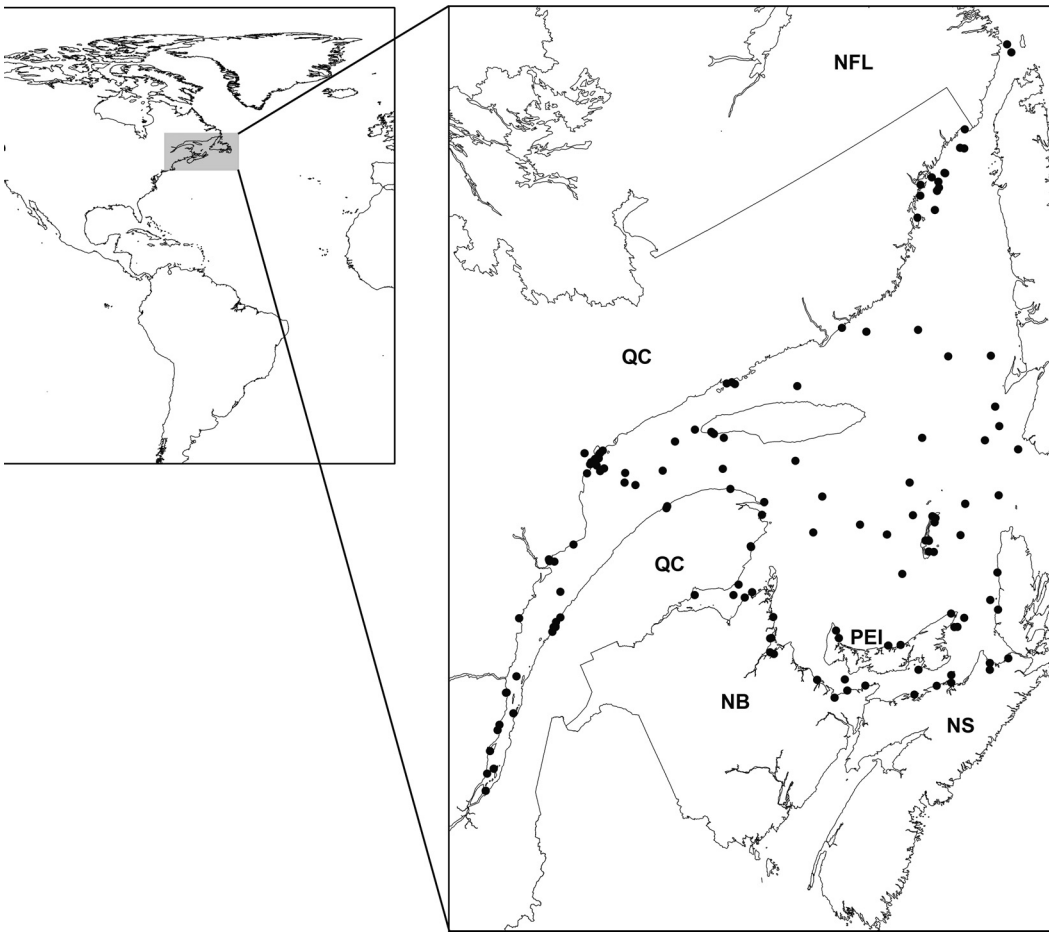

Fig. 1 Distribution map for all sampling sites within the Estuary and Gulf of the St Lawrence River. Canadian provinces surrounding the study area: Québec (QC), New Brunswick (NB), Nova Scotia (NS), Prince Edward Island (PEI), Newfoundland and Labrador (NFL).

molecular phylogenies have challenged systematics at the family and genus levels (Englisch *et al*. 2003; Browne *et al*. 2007; Hou *et al*. 2007). Morphological identification of crustaceans can be difficult, time-consuming and very often requires highly trained taxonomists. Previous work on crustaceans found DNA barcoding to be a useful tool for specimen identification in both marine and freshwater species (Bucklin *et al*. 2007; Costa *et al*. 2007).

This study builds on previous barcoding work on crustaceans by focusing on marine species from the Estuary and Gulf of the St Lawrence River. This geographical region of Atlantic Canada is known for its complexity, having such a wide range of physiographic, oceanographic and bathymetric characteristics that Brunel *et al*. (1998) divided it into 20 biogeographical zones. Although some 770 crustacean species are known from the Estuary and Gulf (Brunel *et al*. 1998), we chose to focus mainly on amphipods and decapods. The former represents the most speciose crustacean order and is an important component of marine food webs. The latter includes species (lobster, shrimp and crabs) that are important economically in providing large harvests and high income to Atlantic Canada, and ecologically as top predators in the marine benthic ecosystem. Our study adds to existing databases a large number of specimens sampled across a vast geographical area for a better representation of intraspecific variation. DNA barcodes reported in this study represent permanent species tags that will not change during taxonomic revisions.

Materials and methods

Samples

We used 507 crustacean specimens collected in the Estuary and Gulf of the St Lawrence River in 2000 ($n = 7$) and between 2005 and 2008 (Fig. 1). The specimens represented 87 described species in 60 genera, 39 families, 5 orders (Amphipoda, Decapoda, Euphausiacea, Isopoda, Mysida) and 1 class (Malacostraca). Deep-water specimens were collected during trawl surveys conducted by Fisheries and Oceans Canada (DFO), while littoral specimens were collected at low tide using dip nets and baited traps. Samples were stored in

100% ethanol (2005–2008) or in 70% ethanol (2000). Morphological identifications were done by experts or followed available keys for North Atlantic amphipods (Bousfield 1973), decapods (Squires 1990), isopods (Schultz 1969), mysids (Brunel 1960) and euphausiids (Mauchline 1971). Scientific names followed the Integrated Taxonomic Information System (www.itis.gov) and the list of McLaughlin *et al*. (2005). In most cases, the whole specimen was stored as a morphological voucher for future reference (Table S2, supporting information). For a few large decapod species, we obtained only tissue (legs or abdominal muscle) for barcoding and we stored these samples as tissue vouchers. However, additional specimens of each of these decapod species have been stored as proper morphological vouchers. In a few juvenile amphipods and crab larvae, no voucher could be preserved due to very small body size, but photographs were taken prior to DNA extraction. All details regarding taxonomy, vouchers and collection sites with geographical coordinates can be found in the Barcode of Life Data System website (BOLD, www.barcodinglife.org) under the 'Crustaceans of the St Lawrence Gulf' project (WWGSL) by following 'View all records'–'Specimen Page' (Ratnasingham & Hebert 2007). In order to insure a geographical coverage for DNA barcodes, when possible, we included multiple specimens (at least two per site) from different geographical areas of the Gulf of St Lawrence (e.g. north shore vs. southern Gulf).

DNA extraction, amplification, sequencing

Laboratory operations were carried out at the Canadian Centre for DNA Barcoding (CCDB), University of Guelph. Total genomic DNA was extracted from small amounts of tissue (1-mm^3 muscle tissue or whole legs for small specimens) by using an automated silica-based protocol with glass fibre filtration plates (Ivanova *et al*. 2006). The barcode region was amplified with alternative sets of primers depending on the reaction success: LCO1490/HCO2198 (Folmer *et al*. 1994) with M13 tails, CrustDF1 (5'-GGTCWACAAA YCATAAAGAYATTGG-3') — CrustDR1 (5'-TAAACYTC AGGRTGACCRAARAAYCA-3') (D. Steinke, University of Guelph, in preparation) and CrustF1/HCO (Costa *et al*. 2007). All primer sequences can be found in the BOLD website within the WWGSL project ('View all records'–'Sequence Page' for each specimen). The polymerase chain reaction (PCR) was performed in 12.5 μL volume containing 2 μL H_2O, 6.25 μL 10% trehalose, 1.25 μL 10× PCR buffer, 0.625 μL $MgCl_2$ (50 mM), 0.0625 μL dNTPs (10 mM), 0.06 μL Platinum *Taq* polymerase (Invitrogen), 0.125 μL of each primer (10 μM) and 2 μL DNA template. PCR thermal conditions included: 1 min at 94 °C, five cycles of 94 °C for 40 s, 45 °C for 40 s and 72 °C for 1 min, followed by 35 cycles of 94 °C for 40 s, 51 °C for 40 s and 72 °C for 1 min, and a final step of 72 °C for 5 min. PCR products were visualized on 96-well precast 2% agarose gels (Invitrogen E-Gel 96 system) and bidirectionally sequenced with BigDye version 3.1 on an ABI 3730xl DNA Analyser (Applied Biosystems). Primers used for sequencing depended on those used for amplification, namely M13 F/M13R, CrustDF1/CrustDR1 or CrustF1/HCO. Additional details about laboratory protocols for each step are available from the CCDB website (www.dnabarcoding.ca).

Data analysis

DNA sequences were aligned with SeqScape version 2.1.1 (Applied Biosystems) and manually checked for ambiguities. DNA sequences as well as trace files are available in the BOLD website within the WWGSL project ('View all records'–'Sequence Page' for each specimen) and on GenBank (Accession nos FJ581463–FJ581922). A BLAST search including one sequence per species was performed on GenBank (megablast algorithm). The Kimura 2-parameter (K2P) model for base substitution (Kimura 1980) was used by BOLD to obtain pairwise genetic distances. A neighbour-joining tree (NJ) based on K2P distances was also built by BOLD for a graphic representation of intraspecific distances. MEGA 4 (Tamura *et al*. 2007) was used to test the NJ tree by bootstrap analysis with 1000 replications. Genetic distances between specimens were calculated for each taxonomic level with the 'Distance Summary' command implemented by BOLD. Cases of intraspecific divergence higher than 3% were considered as potential cryptic species.

Results

Successful amplifications of the barcode region were obtained for 82 out of the 87 species sampled for this study. Amplification failed in the seven specimens stored in 70% ethanol, representing the amphipods *Dyopedos monacanthus* ($n = 1$), *Gammarellus homari* ($n = 1$), *Gammarus fasciatus* ($n = 1$), *Gammarus lacustris* ($n = 2$), and *Jassa marmorata* ($n = 2$). The remaining 500 specimens yielded a positive amplification of COI. Short or low-quality sequences (double peaks, background noise) obtained from 36 specimens and possibly representing pseudogenes were discarded. Only 25% of our sequences had matches in GenBank due to the fact that most species in our study had not been COI-sequenced before. Additionally, the amphipod *Stegocephalus inflatus* ($n = 2$) and the isopod *Calathura brachiata* ($n = 2$) did not match crustacean COI sequences, possibly due to contamination. One discrepancy appeared between our morphological identifications and GenBank: COI sequences of amphipod specimens in poor condition that we identified as *Marinogammarus obtusatus* matched those of the invasive species *Echinogammarus ischnus*.

The database resulting from this study includes DNA sequences for 460 specimens belonging to 80 species and 56 genera. The number of COI sequences per species varied between 1 and 29 with a mean of 5.75. The 658-bp COI

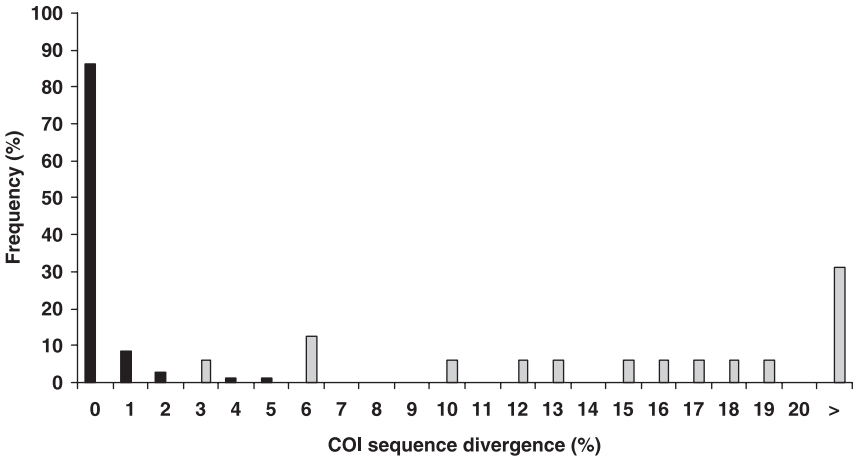

Fig. 2 Frequency distribution of mean divergences for COI sequences (Kimura 2-parameter model) for 80 species of malacostracan crustaceans from the Gulf of St Lawrence. Two taxonomic levels are represented: species (solid bars) and genus (shaded bars). For maximum intraspecific divergences higher than 3% see Table 1.

Table 1 Crustacean species with intraspecific COI sequence divergences higher than 3%

	Species name	No. of intraspecific lineages	Maximum intraspecific divergence (%)	Bootstrap values
1	*Ampelisca eschrichti*	2	13.6	99/99
2	*Ischyrocerus anguipes*	2	4.24	99/94
3	*Neomysis americana*	2	3.78	99/99
4	*Spirontocaris spinus**	3	6.91, 6.41, 3.6	99/98/—

*This species has three lineages, one represented by a single specimen (therefore, no bootstrap support).

fragment had 432 variable sites and 226 conserved sites, while 419 sites were parsimony-informative. Ambiguities were present in a few cases but they did not change the final result. The mean intraspecific divergence was 0.91% while the maximum reached 13.6% (Table S1, supporting information). By contrast, the minimum interspecific distance was 2.81%, resulting in a generally small overlap between the two levels of variation (Fig. 2). Morphological species were represented by individual clusters containing highly similar sequences in 95% of cases (Fig. S1, supporting information). However, four cases of deep intraspecific divergence, greater than 3%, were observed and the respective clades were considered to be potential cryptic species (Table 1; Fig. 3). With these clades removed, the mean intraspecific divergence is 0.51%. The crab larvae sequenced in this study matched *Chionoecetes opilio* sequences, a result confirmed by rearing a few larvae in the laboratory.

Discussion

This study further supports the validity of DNA barcoding for species identification in marine crustaceans. The ratio of interspecific to intraspecific variation (25×) was much higher than the threshold (10×) proposed by Hebert *et al*. (2004) as a species boundary. Therefore, assigning specimens to species was usually straightforward with no overlap between intra- and interspecific distances (95% of cases).

In four morphological species COI sequences grouped into 2–3 clusters that diverged by at least 3% (Table 1; Fig. 3), suggesting either the presence of cryptic species or nuclear mitochondrial pseudogenes (numts). A growing concern regarding numts and DNA barcoding is that, if undetected, numts might lead to an overestimation of species richness (Song *et al*. 2008). In crustaceans, numts have been found to diverge from the COI gene by up to 18.8% (Williams & Knowlton 2001). To investigate the possibility of having amplified numts, we used a few steps suggested by Song *et al*. (2008). We found no stop-codons (quality control tool on BOLD) or indels, the sequences were of high quality, had the expected length (658 bp), matched COI sequences in GenBank, and the proportion of adenine-thymine did not differ strikingly among lineages. Moreover, intraspecific clusters were not related to geography. Consequently, we suggest that the amphipods *Ampelisca eschrichti* and *Ischyrocerus anguipes*, the mysid *Neomysis americana* and the decapod *Spirontocaris spinus* represent species complexes. Classical taxonomy has already inferred the existence of species complexes in North American *Ampelisca* spp. and *I. anguipes* based on the existence of size morphs or subtle differences in morphology (Kaïm-Malka 2000; King & Holmes 2004; references therein). Additional taxonomic, ecological and molecular work is required to investigate the full extent of cryptic speciation in crustaceans from the Gulf of St Lawrence, as DNA barcoding can only serve to flag such cases.

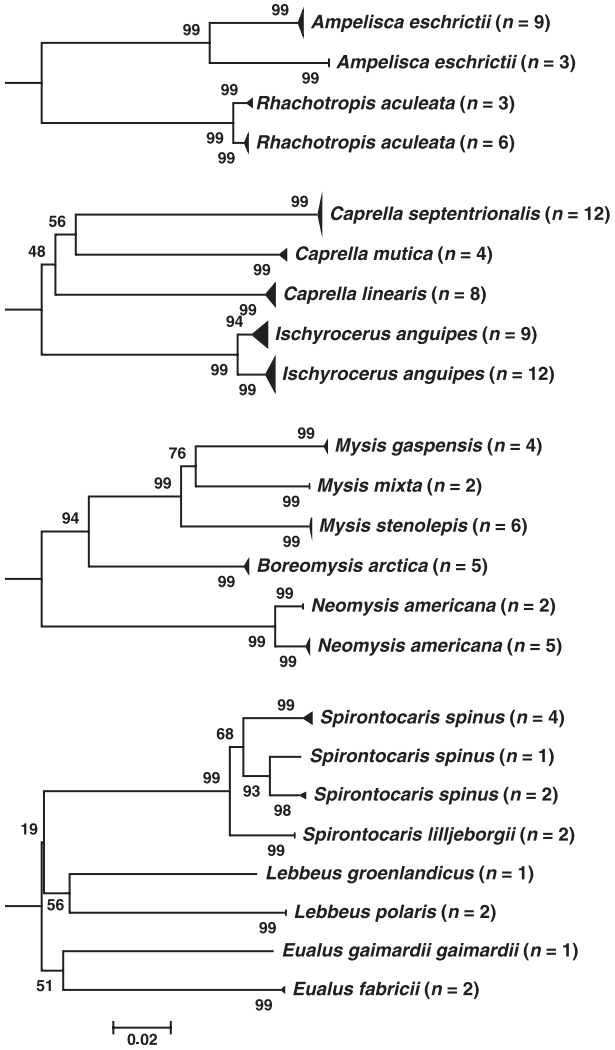

Fig. 3 Branches of the neighbour-joining tree (Kimura 2-parameter model) highlighting the four species complexes (and related species) found in malacostracan crustaceans from the St Lawrence Gulf. Bootstrap values based on 1000 replications are included.

The smallest divergence between species was 2.81% in *Hyas araneus* and *H. coarctatus*, two species that are morphologically distinct from the larval stages to adulthood but genetically close (Hultgren & Stachowicz 2008). This finding is in agreement with other cases of DNA barcoding difficulties for arthropod identification (Barber & Boyce 2006; Burns *et al*. 2007), suggesting once more that the 3% cut-off in sequence divergence is not always applicable and that caution must be exercised in cases of incomplete lineage sorting.

Practical applications of DNA barcoding of crustaceans include detection of invasive species, substitution in processed seafood and estimation of stock size of harvested species based on larval abundances (Costa *et al*. 2007). We report here the presence of an invasive amphipod, *Echinogammarus ischnus*, in the St Lawrence Estuary near Berthier-sur-Mer. This species has spread from its native Ponto-Caspian region into Western Europe and the Great Lakes of North America. In Canada, it has been previously reported along the St Lawrence River upstream from Montréal (Palmer & Ricciardi 2004) and the present study confirms its northeastern expansion. This species was identified as the morphologically similar *Marinogammarus obtusatus* based on specimens in poor condition, but all sequences matched those of *E. ischnus* determined in a previous phylogeographical study (Cristescu *et al*. 2004). Without these reference sequences, our error might have gone unnoticed, thus emphasizing the importance of classical taxonomy to barcoding. Reciprocally, this example also stresses the success of DNA barcoding in rapidly detecting invasive species.

The 80 species sequenced in the present study represent only 20% of about 400 species inventoried within the Estuary and Gulf of the St Lawrence River (Brunel *et al*. 1998) for the five malacostracan orders represented here. Some 20 other amphipod species were not included due to uncertain morphological identifications. Full taxonomic coverage of the known crustacean species from the Estuary and Gulf is hampered by sampling difficulties. Indeed, except for decapods of economic importance (60% sequenced), other malacostracan species are not targeted by regular sampling surveys and seldom show up as by-catch. Moreover, for some taxa (e.g. amphipods), the use of dip nets, baited traps or bottom trawls will lead to a sampling bias towards highly mobile species. Therefore, the fraction of species diversity representing the most common (Brunel *et al*. 1998) and most mobile (Sainte-Marie & Brunel 1985) forms was explored in this study. There are two possibilities to create a comprehensive database for the Gulf crustaceans in the future: research cruises targeting rarer crustaceans or technological advances for high-throughput DNA extraction from formalin-preserved crustaceans. Exploiting museum collections, one of the goals of DNA barcoding, is a difficult task when working with crustaceans due to the traditional use of formalin which negatively affects DNA recovery. Consequently, barcoding studies are most successful when performed on groups that can make use of museum 'dry' collections (e.g. insects, birds, mammals). There is no global campaign yet to barcode all crustacean species (or at least Malacostraca) as exists for other animal groups (e.g. fish, birds, lepidopterans); however, building regional databases throughout the world will bring us closer to understanding crustacean diversity.

In summary, DNA barcoding is a very useful tool for the identification of malacostracan crustaceans by assigning unknown specimens to known species, insofar as species assignations in GenBank are reliable. DNA barcoding may lead to species discovery by flagging cryptic species, although more data than COI sequences are necessary for describing a new species. However, based on DNA barcoding of the most common species at the regional scale of the Estuary

and Gulf of St Lawrence, cryptic species do not appear to be very common.

Acknowledgements

This research was supported through funding to the Canadian Barcode of Life Network from NSERC, Genome Canada (through the Ontario Genomics Institute) and other sponsors listed at www.bolnet.ca. Financial support for fieldwork in the Magdalen Islands was provided by Centre de recherche sur les milieux insulaires et maritimes. We are indebted to the following people for providing specimens: Mikio Moriyasu and Marcel Hébert (DFO Gulf Region), Diane Archambault and the crews on 'Teleost' and 'Calanus', Valérie Bélanger and Gesche Winkler (Institut des sciences de la mer de Rimouski), Annick Drouin (Laval University), the Saunders group (University of New Brunswick, Fredericton), and also to Pierre Brunel (Université de Montréal), Robert Chabot (Université du Quebec à Rimouski) and David Wildish (DFO Maritimes Region) for providing taxonomic support. We thank Traian Brad for valuable field assistance. We are grateful to the CCDB team in Guelph, especially to Janet Topan for laboratory assistance, Natalia Ivanova for help with laboratory protocols and SeqScape, the IT support team, Dirk Steinke for providing primers, Mehrdad Hajibabaei for access to the CCDB facilities and Paul Hebert for his interest in this project. We acknowledge three anonymous reviewers for providing helpful comments which improved the quality of this manuscript.

Conflict of interest statement

The authors have no conflict of interest to declare and note that the funders of this research had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Neighbour-joining tree (Kimura 2-parameter model) for 460 specimens included in this study.

Table S1 Species list with details about mean and maximum intraspecific divergence, nearest neighbour distance and sample size

Table S2 Specimen list including Process ID within the WWGSL project on BOLD (Barcode of Life Data Systems)

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