Rapid and Cost-Effective Differentiation of the Lobsters *Homarus americanus*, *H. gammarus* and Their F1 Hybrids Using DNA-Based Methods

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Article

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Abstract

The American lobster (Homarus americanus) is a non-native species to Europe, but is imported as live seafood, and have been identified in European waters. These introductions threaten native populations of the European lobster (Homarus gammarus) via disease introduction, competition, direct predation, and genetic introgression. Differentiating the two species and their hybrids based solely on morphological criteria can be difficult and unreliable. This study presents a real-time PCR assay targeting the cytochrome c oxidase gene 1 (COX1) for rapid detection and identification of H. americanus and H. gammarus. We have also designed a conventional duplex PCR from a previously described nuclear marker (Hgam98 microsatellite) which was sequenced and revealed the presence of a specific H. americanus insert downstream from a variable number tandem repeat region. The combination of these assays resulted in the accurate identification of the two lobster species as well as F1 hybrid specimens.

Keywords: *americanus*; *gammarus*; *Homarus*; hybrid

1. Introduction

The international trade and transport of live animals can result in species being introduced accidently or maliciously to countries outside their native range. When this occurs, they are classified as a non-native species (NNS). If NNS reach the wild non-native range, either intentionally or accidentally, they may establish themselves, become invasive, and negatively impact local biodiversity. Invasive NNS disrupt ecosystems by introducing disease, preying on native species, and outcompeting them for food, territory, and mates [1]. Additionally, the economic burden of managing non-native aquaculture introductions in the UK has risen sharply, with costs increasing by 139.5% annually to £18 million [2], highlighting the growing strain on national resources.

American lobsters (*Homarus americanus*) are a non-native species to Europe, and are imported as live seafood, but they have been detected in the wild. They can carry pathogens such as gaffkaemia [3] which threaten European lobsters (*Homarus gammarus*), evidence also suggests that *H. americanus* prey on *H. gammarus* [4]. Furthermore, there are concerns about interbreeding *between H. gammarus* and *H. americanus*, which can result in hybrid offspring [5]. Effective regulations and monitoring are essential to protect local ecosystems from both intentional and accidental invasions [6]. Rapid and accurate methods to identify NNS and trace their origins are urgently needed. These tools would enable the implementation of movement restrictions, eradication measures, and, where appropriate, prosecutions against those endangering native biodiversity.

Previously, identification of *H. americanus* and *H. gammarus* has been carried by morphological criteria such as exoskeleton colouration, and presence/absence of the ventral spines on the rostrum. However, it has been demonstrated that identification based solely on morphological criteria can lead to potential misidentification [7–9]. Another method of identification is by polymerase chain reaction

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(PCR) of specific loci which produce different sized products [9], these regions' sequences are currently not published, and so the reason for the differences is uncertain, potentially limiting the accuracy of its use in speciation. Most recently there have been advances in single nucleotide polymorphism (SNP) array analysis [7], which can differentiate *H. gammarus*, *H. americanus,* and determine the likelihood of a specimen being a hybrid. However, this method requires specific equipment that not all laboratories have access to and is better suited to definitive analysis of pre-flagged sample batches rather than ad-hoc screening and/or small-scale sampling due to cost per sample.

Here, we outline the process of sequencing the Hgam98 locus, a previously described diagnostic nuclear locus [9], and the COI gene for possible primer design locations. Since COI is mitochondrial, a COI-based assay would detect any purebred American lobsters (both male and female). *Homarus* species tend to prefer conspecific [10], and the chances of European females finding only American males in their local environment seems unlikely, whereas American females may find dominant European males. This would mean the likelihood that a hybrid animal would have American mitochondrial DNA. However, it is worth noting that American male parentage is also possible, which would not be detected by this assay. During the design process of this project, two animals were discovered off the coast of Cornwall (50°18'44.1"N 4°40'09.0"W), displaying potential *H. americanus* and *H. gammarus* mixed morphologies, which were also assessed for hybridisation, highlighting the importance of molecular analysis over morphological identification.

2. Materials and Methods

2.1. Biological Material and DNA Extraction

Tissues of American and European lobsters were collected from adults sourced from their native ranges (Table 1), via the sub-lethal excision of pleopod and/or pereiopod sections [9,11]. Hybrids of the two species were collected as the larval offspring of an American female captured in Sweden and fertilised by a European male, as ascertained by previous research [9]. All samples were preserved in absolute ethanol and stored at -20 °C, prior to DNA being isolated via a modified salting-out protocol [11]. DNA from six individuals from each of these European, American and hybrid lobster cohorts were obtained (Table 1).

Table 1. Identities of various lobster samples, their locations of origin, and number of their smallest VNTR product. HAM, AxG, and GAM supplied by University of Exeter. Corn samples supplied by IFCA.

Identification	Species	Location	Number of VNTRs (A[C/T]AG)	Accession number
HAM1	*H. americanus*	Browns Bank, Massachusetts, USA	17	PQ106807
HAM2	*H. americanus*	Caraquet, New Brunswick, Canada	18	PQ106808
HAM3	*H. americanus*	Coastal New Hampshire, USA	5	PQ106809
HAM4	*H. americanus*	Fortune Bay, Newfoundland and Labrador, Canada	17	PQ106810
HAM5	*H. americanus*	Malpeque Bay, Prince Edward Island, Canada	26	PQ106811
HAM6	*H. americanus*	Musquodoboit Harbour, Nova Scotia, Canada	26	PQ106812
AxG1	Hybrid animal	Lysekil, Sweden	15	PQ106813

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AxG2	Hybrid animal	Lysekil, Sweden	15	
AxG3	Hybrid animal	Lysekil, Sweden	15	
AxG4	Hybrid animal	Lysekil, Sweden	15	
AxG5	Hybrid animal	Lysekil, Sweden	15	
AxG6	Hybrid animal	Lysekil, Sweden	15	
GAM1	*H. gammarus*	Bergen, Norway	15	PQ106814
GAM2	*H. gammarus*	Flodevigen, Norway	15	
GAM3	*H. gammarus*	Lysekil, Sweden	15	PQ106815
GAM4	*H. gammarus*	Tangiers, Morocco	15	PQ106816
GAM5	*H. gammarus*	Tangiers, Morocco	15	
GAM6	*H. gammarus*	Tangiers, Morocco	15	
Corn1	Unknown	Cornwall, UK	15	N/A
Corn2	Unknown	Cornwall, UK	15	N/A

Additionally, pleopod tissues were supplied from two adult lobsters captured in Cornwall, U.K., by the Inshore Fisheries and Conservation Authorities (IFCA); these lobsters both exhibited the presence of a sub-rostral 'tooth' and reddish coloured spines, traits typically associated with American lobsters, albeit with broader morphologies characteristic of the European lobster.

DNA from these two samples was extracted by taking 0.1 g of tissue in a 1:10 dilution of G2 buffer and proteinase K at 0.2ug/ml (Qiagen) into a 2 ml lysing matrix A tube (MP biomedicals). The contents of these tubes were then subjected to mechanical disruption using a fast-prep 24 classic (MP biomedicals) at 4 m/s for sixty seconds before incubation overnight at 56 °C. Digests were then subject to extraction with the DNA tissue kit (Qiagen) via an EZ1 Extraction robot (Qiagen) and the nucleic acid was eluted into a 50 µl volume.

2.2. DNA Amplification and Sequencing

To amplify the Hgam98 locus, polymerase chain reaction (PCR) was performed in a 50 µl volume containing 2.5 µl of the DNA elution, 0.5 µl of 25 mM dNTPs, 10 µl of 5x green GoTaq flexi buffer, 5 µl of 25 mM $MgCl_2$, 0.25 µl of GoTaq G2 Flexi DNA Polymerase (Promega), 0.5 µl of forward and reverse Hgam98 primers at 10 µM (Table 2) and made up to 50 µl with molecular biology grade water. The cycling program had an initial denaturation at 94 °C for five minutes, followed by 35 cycles of 94 °C for one minute, 55 °C for one minute and 72 °C for one minute, with a final extension of 72 °C for ten minutes. PCR products were resolved on 3% agarose gel (w/v) agarose/TAE (40 mM Tris-acetate, pH 7.2, 1 mM EDTA) containing 1.0 µg/ml ethidium bromide, at 120 V for thirty minutes. Products of the expected size were excised, and the DNA purified using spin modules (MP Biomedicals). Both strands of the DNA were sequenced by Sanger sequencing using Big Dye Terminator 3.1 methodology (Life Technologies) using the manufacturer's recommended protocol and the sequences were analysed on a 3500xl genetic analyser (Applied Biosystems) and consensus sequences generated using CLC workbench 24 software (Qiagen). Contigs were then aligned and compared using MEGA 7 software [12].

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Table 2. Primers used in this experiment, their sequences, and their origins.

Primer/Probe name	Primer sequence	Target organism	Reference
Hgam98 F	GCCTCCGTCGGGTTTCCTG	*H. gammarus*/*H. americanus*	Jørstad et al 2007 (modified)
Hgam98 R	AATATGTCTTTCAATGGCTTCTCC	*H. gammarus*/*H. americanus*	Jørstad et al 2007 (modified)
Hamer F	ATAACGAGATAGTGTAGGG	*H. americanus* (Insert)	This study
COI_H_SP_F	ACTGGRTGAACTGTCTACCC	*H. gammarus*/*H. americanus*	This study
COI_H_SP_R	CCAGCYAGATGAAGCGAGAA	*H. gammarus*/*H. americanus*	This study
COI_HA_Pr	[6FAM] CTCACGCAGGTGCTTCCGTTGA [TAM]	*H. americanus*	This study
COI_HGam_Pr	[HEX] CGCTTCTGTTGATTTAGGAATTTTCTCGCT [TAM]	*H. gammarus*	This study

2.3. Duplex Conventional PCRs

Using the sequencing data from the Hgam98, an additional primer (HAMER) was designed located in the *H. americanus* insert (Table 2 and Figure 1), which was combined with the primers of the Hgam98 assay. This PCR was conducted using the same method as above, but with the addition of 0.5 µl of the HAMER F primer.

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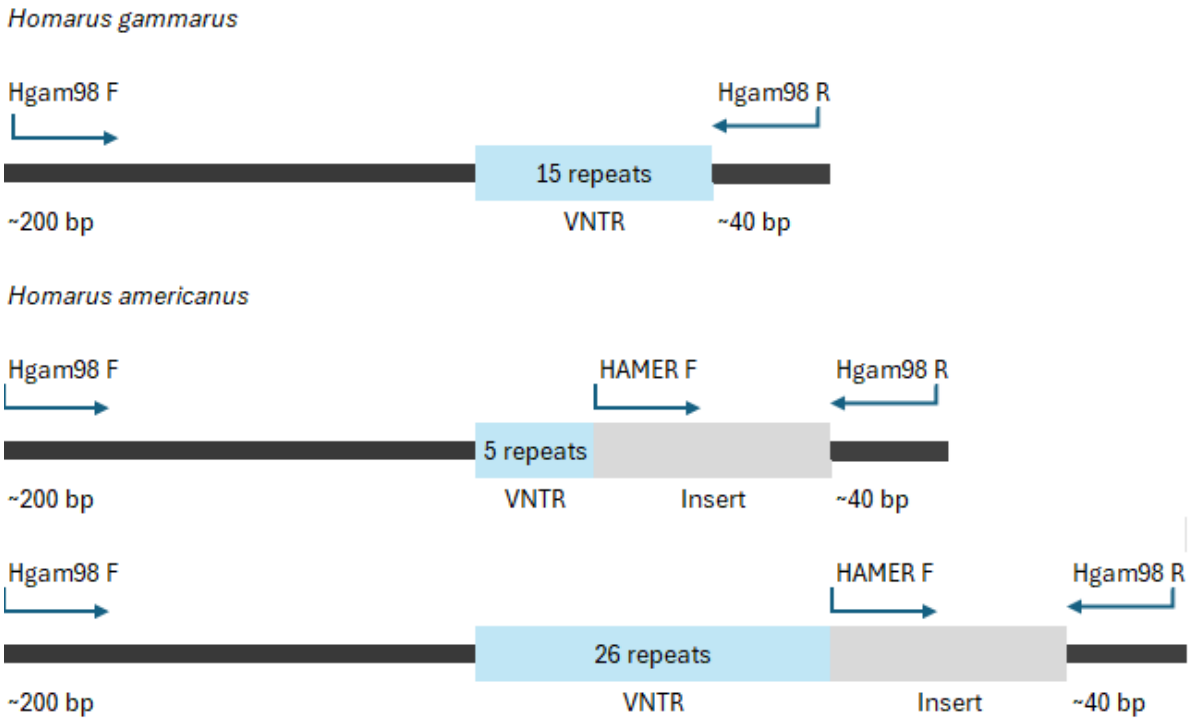

Figure 1. A diagram of amplicon layout, highlighting the cause of the size discrepancies (H. americanus shown with minimum/maximum repeats) and the location of PCR primers.

2.4. Real-Time PCR Design and Application

A Real-Time PCR assay was developed targeting the Cytochrome c oxidase subunit I gene (COXI) since regions of this gene can be used to identify *H. americanus* and *H. gammarus*. The primer pair COI_H_SP_F/R (Table 2) was designed to amplify a 90 bp fragment of COI. Primers and probes were designed *in silico* by aligning sequences obtained from Blastn search engine (Basic Local Alignment Search Tool [13]). The real-time assays were performed in a 20 µl duplex reaction volume containing 2.5 µl of DNA template extracted from American and European lobster samples 10 µl of TaqMan universal master mix (Life technologies), 1 µl of each primer and probe, and made up to 20 µl with molecular grade water. Assays were performed on a Quantstudios 3 thermocycler (Applied Biosystems) using cycling settings with an initial hold stage of 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 15 seconds, and 62 °C for 1 minute. Each plate included two negative controls (NEG) to ensure the absence of contamination.

3. Results

3.1. Sequencing the Hgam98 Locus

The Hgam98 PCR products showed the expected size for *H. americanus* (~440 - 500 bp) and *H. gammarus* (~290 - 330 bp) (Figure 2). The sequencing of six *H. americanus* and six *H. gammarus* animals showed a segmental similarity where *H. americanus* and *H. gammarus* share an identical 5` 211 bp region before diverging into variable nucleotide tandem repeat (VNTR) regions. In *H. gammarus* samples, the number of repeats remains mostly the same whereas *H. americanus* samples showed a greater range of sizes (Table 1). Additionally, *H. americanus* has a 120 bp insert that is absent in *H. gammarus*. The two species then reconverge with an identical 40 bp sequence at the 3` end (Figure 2). In the case of hybrids, alleles with the expected size for *H. americanus* and *H. gammarus* were observed

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in some of the individuals genotyped (lanes 10 and 11). However, for other individuals very faint or no bands were observed for Hgam98 (lanes 7, 8, and 9), but a clear band with the expected size was observed using the HAMER primer (lane 7 to 12).

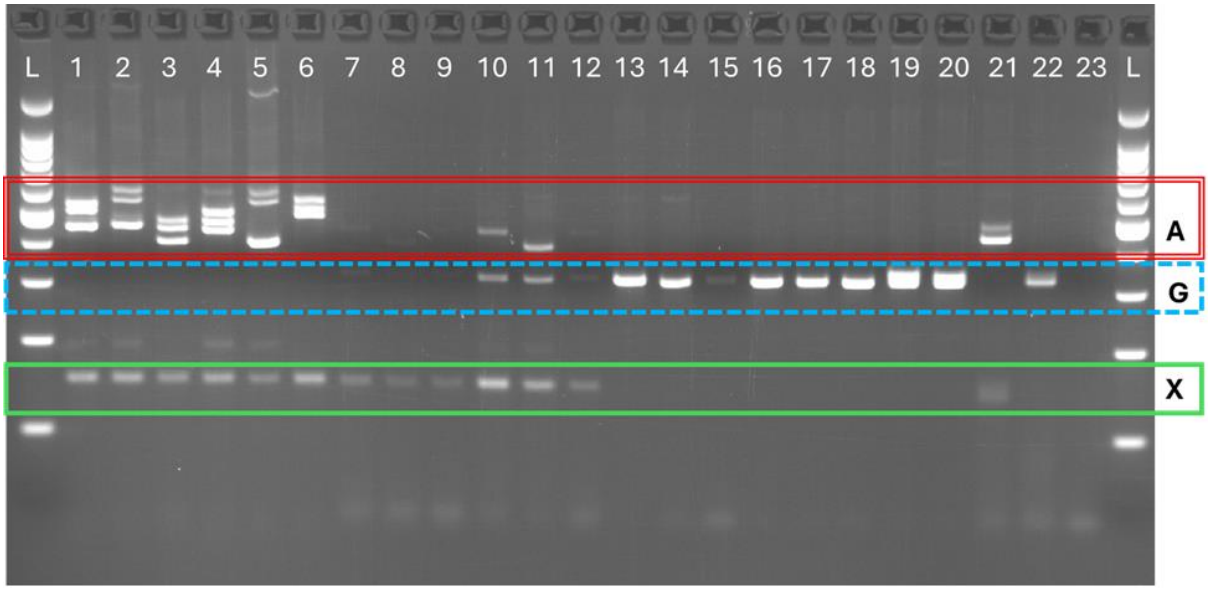

Figure 2. A gel image showing the results from the Hgam98 duplex assay. Bands can be seen in the H. americanus section (A), H. gammarus section (G) and the HAMER98 H. americanus only product (X). Lanes 1-6 show HAM samples, 7-12 show AxG samples, 13-18 show GAM samples, 19 and 20 show Cornish IFCA samples, lane 21 shows known H. americanus, lane 22 shows known h gammarus, and lane 23 is a negative PCR control.

3.2. Duplex Conventional PCR

When used in conjunction with the Hgam98 assay, the HAMER primer produced no additional product for *H. gammarus* samples but did produce PCR products of ~150 bp for *H. americanus* (Figure 3). When sequenced, these products showed 100% nucleotide sequence similarity to the *H. americanus* insert. In the hybrids tested, the 152 bp insert was observed, and again, with 100% nucleotide similarity to *H. americanus* insert.

The putative hybrids from Cornwall showed only *H. gammarus* products, and no *H. americanus*, or HAMER products.

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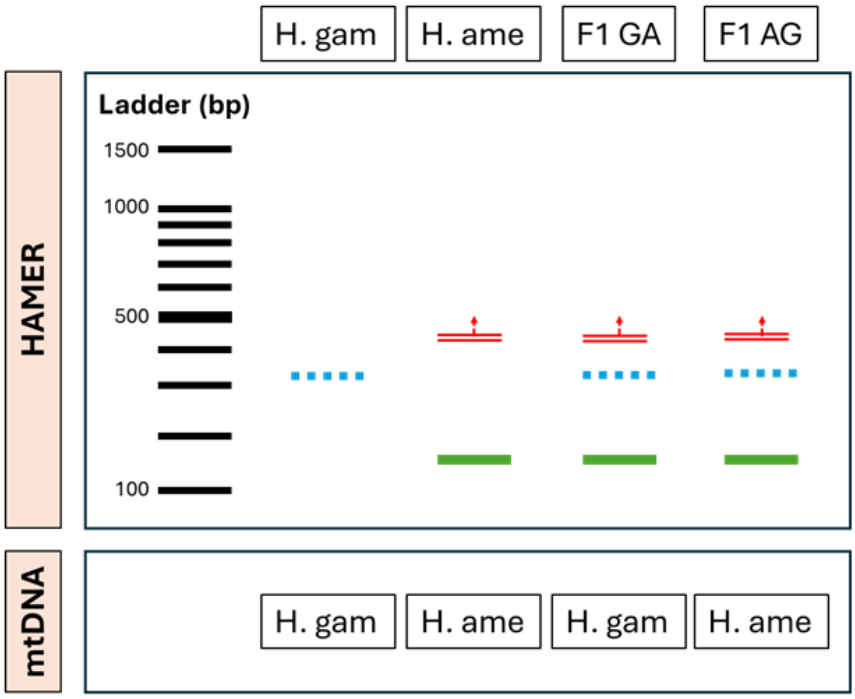

Figure 3. a schematic representation of a gel for the HAMER assay; Green (solid line) denoting the presence of the insert region. Blue (dashed line) denoting the European HGam98 allele. Red (double line) denoting the American HGam98 allele, with arrows to indicate the variability in size.

3.3. Real-Time PCR

The linearity of the assays was checked through the analysis of three replicates of a ten-fold serial dilution of known DNA (10^8 to 10 copies of template). The regression coefficient (R^2) and slope for the *H. americanus* assay was 0.990 and –3.383, respectively. For the *H. gammarus* assay, the R^2 and slope were 0.999 and –3.333, respectively. These results indicate that, within the range of DNA amounts tested, the assays gave reliable results, and both assays could detect positives at 10 copies with Ct values of 34.7 for *H. americanus* and 33.7 for *H. gammarus*.

The potential hybrid material from Cornwall produced only positives with the *H. gammarus* probe. These animals were then subjected to the SNP analysis [9] to confirm results as part of the validation, formally identifying these animals as native European lobsters (*H. gammarus*).

4. Discussion

The results obtained have explained the reason for the product size differences between *H. americanus* and *H. gammarus* when using the HGAM98 PCR assay, this aids in the justification of its use, but highlights limitations as fewer repeats could be misread as a *H. gammarus* positive. The further development of the HAMER duplex protocol helps identify not only NNS American lobsters, but potentially also *H. americanus* X *H. gammarus* F1 hybrids. Additionally, the results also suggest that the Real-Time PCR assay can accurately differentiate between *H. americanus* and *H. gammarus* mitochondrial DNA (mtDNA), with each specific probe detecting its respective species as seen in the real-time graphs. Since the assays could detect DNA at 10 copies and the detections of the hybrid material as *H. americanus* it highlights the potential to detect American maternal F1 hybrid animals rapidly, and more cost effectively on an ad hoc sample basis.

In the case of the lobsters of mixed morphology captured off the coast of Cornwall, all tests identified the individuals as being *H. gammarus*, a determination confirmed by the SNP-based method of Ellis et al [9] (data not presented). This highlights the importance of having additional reliable molecular methods of species determination, since morphological traits are unreliable and

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can show signatures of mixture that cannot be attributed to hybridisation, and SNP-based methods may be too expensive for some authorities to set-up if only sporadic testing is likely.

During this experiment, it was discovered that the salting-out method (Jenkins et al 2019) incurred PCR inhibitors [14], which were mitigated by diluting each sample 1:10 with molecular biology grade water. It is also worth noting that sequence data for hybrids is extremely limited due to their rare occurrence, in this study, all six hybrid animals were full siblings that were hatchery produced. This means there is the possibility that this range of methods may not detect certain types of hybrids, such as *H. gammarus* (female) X *H. americanus* (male) X, or any potential F2 or backcrossed hybrids. However, it is hoped that the methods outlined in this study will not only improve the efficiency and practicality of monitoring for non-native *H. americanus* but also aid in the detection of hybridisation amongst wild lobster stocks. The HAMER duplex protocol adequately detected the hybrid siblings we tested, and the Real-Time PCR assay for COI could provide rapid flagging of hybrids with *H. americanus* maternity, even if they displayed European morphologies. These molecular resources can enhance the toolkit available to monitor the presence of *H. americanus*, *H. gammarus* and F1 hybrids in regions where they might have been introduced, offering rapid and cheap screening compatible with bulk sample processing or routine monitoring.

To surmise, we believe due to the low cost and rapid results, the addition of these assays to the suite of tests, like SNP analysis can greatly improve detection rate in NNS monitoring. This study had a limited sample size, especially with regards the hybrid material, but it is felt this project demonstrates a proof of concept, and future studies with additional hybrid lineages, and qPCR analysis on eDNA samples would further validate the approach.

Author Contributions: Conceptualisation, M.E.; methodology, M.E. and C.E.; resources, M.E. and C.E.; writing: original draft preparation, M.E.; writing: review and editing, C.E. and F.B.; supervision, F.B.

All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was reviewed and granted an exemption by the Animal Welfare and Ethical Review Bodies (AWERB). No live animals were handled and all samples of Homarus americanus and Homarus gammarus were collected post-mortem, and therefore the work did not fall under regulations requiring ethical approval. The study was conducted in accordance with international standards for research integrity and animal welfare (ARRIVE) guidelines.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

COX1	Cytochrome c Oxidase gene 1
NNS	Non-Native Species
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
IFCA	Inshore Fisheries and Conservation Authorities
VNTR	Variable Nucleotide Tandem Repeat

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