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Article

Identification of the Invasive Form of *Corbicula* Clams in Ireland

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Abstract: The basket clam genus, *Corbicula*, commonly known as the Asian clam, has become one of the most internationally high-profile and widespread aquatic invasive species. This genus is now considered to comprise a polymorphic species complex. The international invasion of *Corbicula* is characterised by four lineages, each fixed for one morphotype, genotype and haplotype combination: the American form (A) and European round form (R), the American form (C) and European saddle form (S), American form B, form round light colour (Rlc) and an intermediate between forms R and S known as Int. We investigated the genetic and morphometric makeup of each Irish population in order to establish which invasive lineages were present so as to identify the number of introductions to Ireland. A combination of morphometric, mitochondrial cytochrome oxidase subunit I (mtCOI) gene analysis and microsatellite markers were used to determine the invasive form at each Irish site. All Irish *Corbicula* samples conformed morphometrically to the invasive form A/R. All mtCOI sequences retrieved for 25 Irish individuals were identical to the international A/R form, while microsatellite markers again showed a common clustering with the international A/R forms of *Corbicula*. The combined approach of morphometries, total genomic DNA and microsatellite markers indicate only one form of *Corbicula* invaded Ireland; the international A/R form.

Keywords: Veneroida; biological invasion; Ireland; mtCOI; microsatellites; morphology

1. Introduction

Corbicula is a freshwater bivalve genus, commonly known as basket clams. *Corbicula* clams are native to Asia, Australia, Middle East and Africa, and have become invasive in Europe and the Americas [1–4]. Interestingly, invasive *Corbicula* clams, as well as most native lineages, reproduce through a peculiar and rare asexual reproductive mode, i.e., androgenesis or strict paternal inheritance. Androgenesis in *Corbicula* occurs through the expulsion of maternal nuclear chromosomes after obligatory fertilization of an oocyte by an unreduced biflagellate androgenetic spermatozoa [5–7].

In North America, the first record of an invasive lineage of *Corbicula* was made in 1924 in British Columbia [8], with subsequent spread through many parts of the continent (reviewed in [9]). In addition, *Corbicula* has spread across much of Central and South America during the 1970s and 1980s [3,8–11]. The first records of *Corbicula* in Europe were in 1980 from the River Dordogne in France and the Tagus estuary in Portugal [1]. Subsequently, *Corbicula* has become widespread through much of Western Europe [12] and England [13].

In Ireland, *Corbicula* clams were first detected in the River Barrow in 2010 [14], where an extremely high density of 17,872 individuals/m² recorded at one sampling site in 2012 [15]. A subsequent

investigation revealed its presence in the River Nore [16], which shares a common estuary with the River Barrow. *Corbicula* was also discovered in the Shannon system, Ireland's major navigable waterway, at Carrick-on-Shannon [17], in Lough Derg and in the Shannon River section above Portumna upstream of Lough Derg [18] and at Lanesborough, upstream of Lough Ree (R. Sheehan, Pers Obs) (Figure 1).

Invasive lineages of *Corbicula* clams have previously been described as belonging to two species, *Corbicula fluminea* and *Corbicula fluminalis* but are now assigned to forms [11] based on molecular markers because of the confused taxonomy of the genus and invasive lineages. A morphological taxonomic identification of *Corbicula* lineages has proved problematic as a wide range of shell morphology exists across invaded countries and within their native Asian range [19–24]. The use of mitochondrial analysis and the development of microsatellite markers for invasive *Corbicula* lineages in Europe, has increased our understanding of both invasive and native populations worldwide [11,24].

Internationally only four lineages of *Corbicula* have become invasive, with each being fixed for one morphotype, genotype and haplotype combination [11]: form A/R, form C/S, form B and form Rlc [11]. Form A/R is found in North and South America as well as in Europe, being the most widely distributed invasive lineage [8,11,21,25]. This form's mtCOI haplotype (FW5) has also been recorded in *C. leana* individuals, an androgenetic Japanese lineage [11]. Form R refers to the strict European populations, whereas form A strictly describes the American forms, but both have the same COI haplotype and microsatellite genotype. Form C/S (haplotype FW17) has been recorded both in South America (form C) and in Europe (form S) and appears related to *C. fluminalis* [11,21]. Form B, showing the haplotype FW1, is found in North and South America [21]. Finally, form Rlc is found only in Europe, where its distribution appears restricted to the Rhone basin in France [23,26]. Form Rlc's mitochondrial COI haplotype FW4 is closely related to that of Form B, as they differ only at one nucleotide site but are distinct at the microsatellite genotype [11].

Within invasive populations of *Corbicula*, further genetic and morphometric complexities can arise from these cryptic hybridizations between sympatric, distinct androgenetic lineages (reviewed in [27]). Indeed, when androgenetic reproduction occurs between two different lineages, the offspring are associated with the nuclear genome and thus, the morphology of the paternal lineage combined to the mitochondrial genome of a distinct maternal lineage, as mitochondria are maternally inherited. This association within one individual of the nuclear genetic information of one lineage and the mitochondrial genetic information of another is called a cytonuclear mismatch [28] and leads to cryptic hybrids. From time to time, nuclear chromosomes can be inadvertently retained within the zygote [6] leading to hybrid individuals with intermediate phenotypes between distinct forms. Such cytonuclear mismatch and hybrid individuals have been widely documented in the *Corbicula* invasive range [11,19,21,23,28,29]. A recent form D was described in North America [30] but it could be a hybrid between form A/R and form B, as the morphotype is intermediate between these two forms, the COI is from form A/R [23] and the 28S is found in form B [28], as well as in Asia [31]. Moreover, the environment can influence the phenotype, thus complicating the determination of an invasive form when it is based solely on morphotype. For example, differing *Corbicula* morphotypes in two estuaries in Portugal, possess the same haplotype FW5; their morphological differences are likely due to differing environmental factors in each estuary [22].

The phenomena of cytonuclear mismatches and cryptic hybrids in *Corbicula*, highlight the importance of combining molecular genetic methods (mitochondrial and nuclear markers) with morphological identification as a dual approach to accurately characterize invasive populations [23].

The pathways and mode by which *Corbicula* was introduced and has subsequently spread within Ireland are poorly understood [32], with recreational water activity being one possible explanation [33]. It is unclear whether only one discrete introduction occurred, with populations at other sites resulting from secondary spread or alternatively, if a number of separate introductions took place from outside Ireland to each site. Determining distinct morphotypes, genotypes and haplotypes in the various Irish *Corbicula* populations would support a hypothesis that a number of discrete introductions occurred.

The high level of phenotypic plasticity present within the invasive forms of *Corbicula* [22] and the novel reproductive strategies leading to possible cytonuclear mismatches and cryptic hybrids [23] mean any one method on its own from morphology, mitochondrial and microsatellites is likely to provide an incorrect identification of the invasive form tested. The cross-lineage genetic mixing and recent spread of androgenetic *Corbicula* lineages [27] and the presence of mismatches between mitochondrial and nuclear markers [11,23,28] further demonstrates the need for a multimethod approach.

Aims

This study aimed to demonstrate the effectiveness of combined morphological and genetic approaches (both mitochondrial and nuclear markers) in resolving invasive bivalve identification. The research also assessed the methodology as an invasion-source tool by using *Corbicula* specimens from separate invaded sites in Ireland and Belgium.

2. Materials and Methods

2.1. Specimen Collection

Five sites in Ireland with known populations at the time this study was conducted, were sampled to collect individuals for genetic and morphological study (Figure 1; Table 1). One site in Belgium, in the Meuse River at Petit Lanaye (Table 1) was sampled on the 23/10/2013 to collect individuals solely for morphological comparison with individuals from the Irish sites.

A range of standard sampling methods were used to collect *Corbicula* specimens, depending on water depth and site location, including SCUBA diving, benthic dredge, grab sampler and kick-net. Samples in Ireland were collected from five sites between 2011 and 2013; the Shannon River at Carrick-on-Shannon, Lanesborough, Portumna and Lough Derg, as well as the River Barrow, the River Nore and the Meuse River (Table 1). The Shannon River basin sites were collected from a range of river and lake settings with the St. Mullin's and Red House sites [16] being riverine.

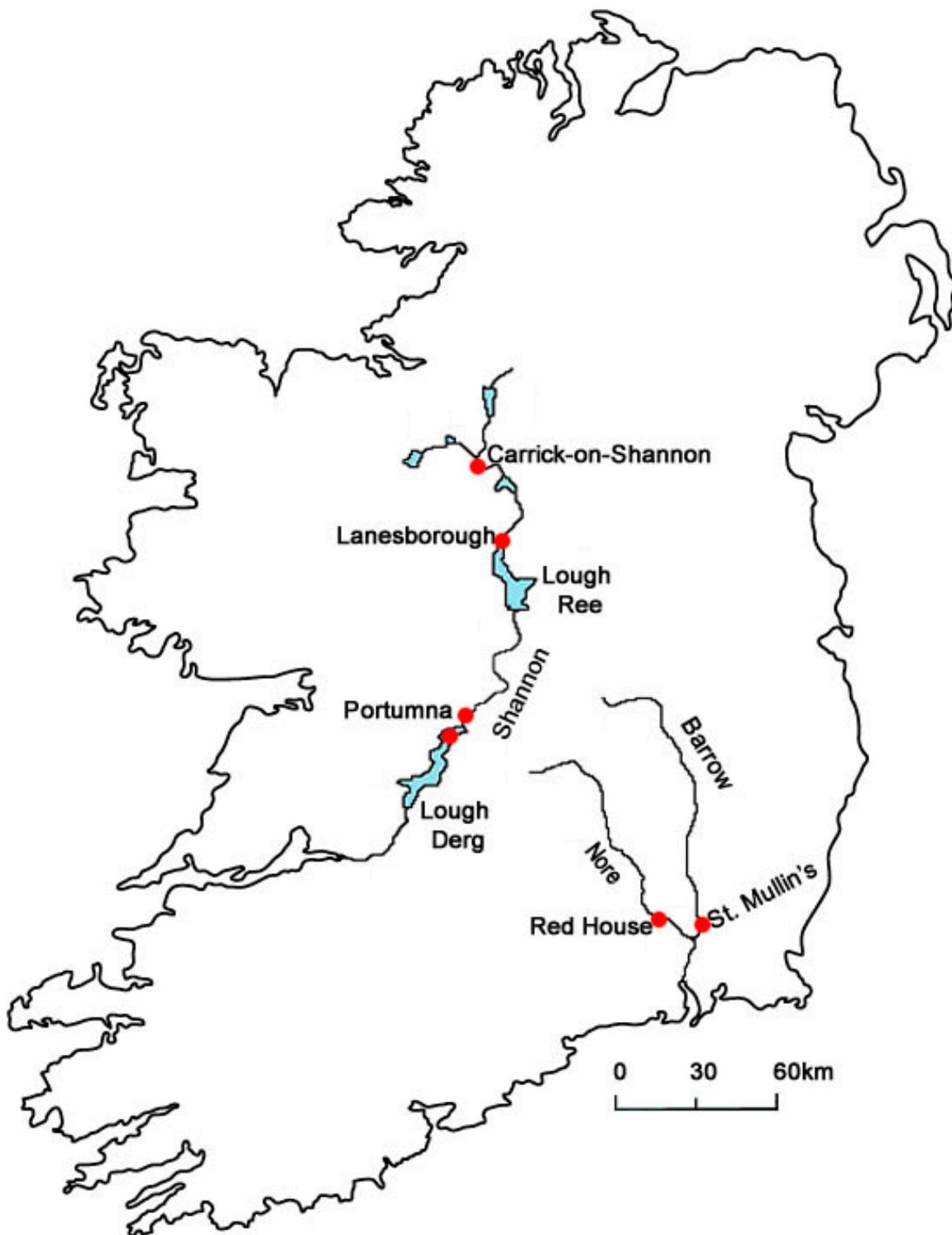


Figure 1. Irish rivers containing *Corbicula* specimens, previously described as *C. fluminea*, sampled during this study, River Nore, Barrow and Shannon. Sampling sites Carrick-on-Shannon, Lanesborough, Portumna, Lough Derg, St. Mullin's and Red House.

Table 1. Sampling sites and collection methods of *Corbicula* clams for DNA and morphometric analysis.

Site	No	Site	Sampling Method	Grid Reference
Shannon (Carrick-on-Shannon)	1	1 km above town	Diving	53.951402, −8.109267
Shannon (Lanesborough)	2	Below bridge	Grab + dredge	53.669457, −7.998963
Shannon (Portumna)	3	Below bridge	Grab + dredge	53.091139, −8.195643
Lough Derg	4	Upper lake	Grab + dredge	52.928985, −8.280803
River Barrow	5	St Mullin's	Diving	52.487025, −6.926753
River Nore	6	Redhouse	Kick-net	52.469403, −7.044622
River Meuse	7	Meuse River, Petit Lanaye, Belgium	Kick-net	50.810898, −5.692482

2.2. Morphological Analysis

A morphological examination of each individual from Ireland (N = 84) and Belgium (N = 51) was carried out, separating them into the morphotypes described in Europe, R, S, Rlc and also, an intermediate morphotype (Int) [19], in order to determine the form of *Corbicula* according to the morphotype descriptions in [21] and [26]. Form R, (round form) has a shell with well pronounced concentric ridges, is round and broad and generally attains a larger size than form S. Its internal colour is white but may contain pallid purple markings [26]. Form S, (saddle form) again has concentric ridges but these are less raised than in the R form and is narrower and proportionately longer. The internal shell colour is a deep purple throughout. Form Rlc (light colour R form) is superficially similar to the R form in shape, but with a lighter surface shell colour and an off-white to yellow internal colour [26]. Form Int is similar in shape to the R form but with finer less pronounced ridges on the shell [19].

All specimens selected from Ireland and the River Meuse were measured to the nearest mm for shell height (H), length (L) and width (W) using a pair of digital calipers to determine individuals of form R, S and Intermediate (Int), Rlc was not found (Table 2). The ratio between each measurement was calculated for the Irish *Corbicula*, and compared to individuals of form R, S and Int from the River Meuse. A Principal Component Analysis (PCA) was conducted in RStudio (Version 0.98.501), on the ratios between shell length, height and width, (L/H, L/W, H/W), as described in [21].

Table 2. Numbers of individuals (forms R, S and Intermediate (Int)) used for the morphological (Ireland and Belgium), mtCOI and microsatellites (Ireland only) analyses.

Site	Described Form	Morphology	mtCOI	Microsatellites
Carrick-on-Shannon	R	11	5	9
Lanesborough	R	9	5	9
Lough Derg	R	39	5	14
River Barrow	R	10	4	9
Portumna	R	10	1	2
River Nore	R	5	5	5
Meuse River	R	10	N/A	N/A
	S	37	N/A	N/A
	Int	4	N/A	N/A

2.3. DNA Extraction

Adductor muscle tissue and foot were dissected from each of the Irish specimens for genetic analysis. The samples for the Carrick-on-Shannon, Lanesborough, River Barrow and River Nore sites

were preserved in 98% ethanol and stored at ambient temperature. Samples from Lough Derg were initially preserved in methanol and transferred to ethanol after dissection.

Total genomic DNA was extracted from the adductor muscles and/or foot of 50 individual specimens (Table 2), using the DNeasy blood and tissue kit (Qiagen), according to the manufacturer's protocol. DNA extraction and microsatellite sequencing were carried out at the Laboratory of Evolutionary Genetics and Ecology, (LEGE), University of Namur, Belgium, as previously described in [11,23].

2.4. Mitochondrial COI Gene Analysis

A fragment of 710 bp of the COI gene was amplified in 25 individuals, with representatives from each Irish location. Polymerase Chain Reaction (PCR) was carried out using the universal primers LCO1490 and HCO2198 [34] following the protocol described in [23]. Amplicons were purified and sequenced with the forward universal primer HCO2198 on an automated ABI 3730XL Genetic Analyzer (Genoscreen, Lille, France). Retrieved sequences were visualized, aligned and edited using BioEdit 7.0.5.3 [35]. Corrected sequences and published sequences of the invasive lineages (FW5—Form A/R: GU721082; FW1—Form B: AF196269; FW4—Form Rlc: GU721084; FW17—Form C/S: GU721083) were added to our dataset and used to construct a haplotype median-joining network using the Network 4.6.1.2. [36].

2.5. Microsatellite Marker Analysis

Ten microsatellite markers [24] (CIA01, CIA02, CIA03, CIB03, CIB11, CIC01, CIC08, CIC12, CIE01, and CID12) were amplified following the protocol of [24]. Microsatellite markers were read on an ABI 3130XL Genetic Analyzer with GeneScan-500 (LIZ) size standard (Applied Biosystems) and scored using GENEMAPPER (Applied Biosystems).

For each of the 50 individuals analysed, we defined a multilocus genotype (here, the unique combination of alleles for the 10 microsatellite loci). These individuals as well as 47 individuals from the European and American invasive individual's lineages previously typed (10 individuals from Form A/R, 16 from Form B, 10 from Form Rlc and 11 from Form C/S) were clustered based on their multilocus genotype using a discriminant analysis of principal components (DAPC) [37]. The DAPC analysis was performed using the package adegenet [38] implemented in R version 2.15.2 (R development core team 2008). The number of putative populations was first determined using the k-means clustering algorithm [39] for $K = 1$ to $K = 11$; 11 being the number of sampled populations added in the analysis. The appropriate number of clusters was defined using the Bayesian Information Criterion (BIC); the value at which the BIC distribution forms an elbow indicating the best clustering (Supplementary Material S1). The relationships between the BIC-defined clusters were then inferred. Six principal components and one discriminant function (98.9% of the total variance) were retained to represent the majority of the variability contained in the dataset.

3. Results

3.1. Morphology

From the morphological characterization, all Irish *Corbicula* clams were visually determined as the European form R as described in [19,23] with a round deep shell that may range from externally dark to golden, heavy ridges and a generally white interior (Figure 2).



Figure 2. Examples of *Corbicula* form R from the River Shannon at Lanesborough (juvenile specimen) and Carrick-on-Shannon and the River Barrow at St. Mullin’s. Also shown is an example of Form S from the River Meuse at Maastricht.

The PCA analysis (Figure 3) of shell height vs length shows the Irish *Corbicula* grouping together with the Belgium form R and the international intermediate (Int) form. The Int form displayed an intermediate morphology between R and S, with the round body shape of R and the closely spaced shell ridges and purple interior coloring of S. The Irish individuals were different from the Belgian form S. No Irish individuals had a shell height/length ratio consistent with form S.

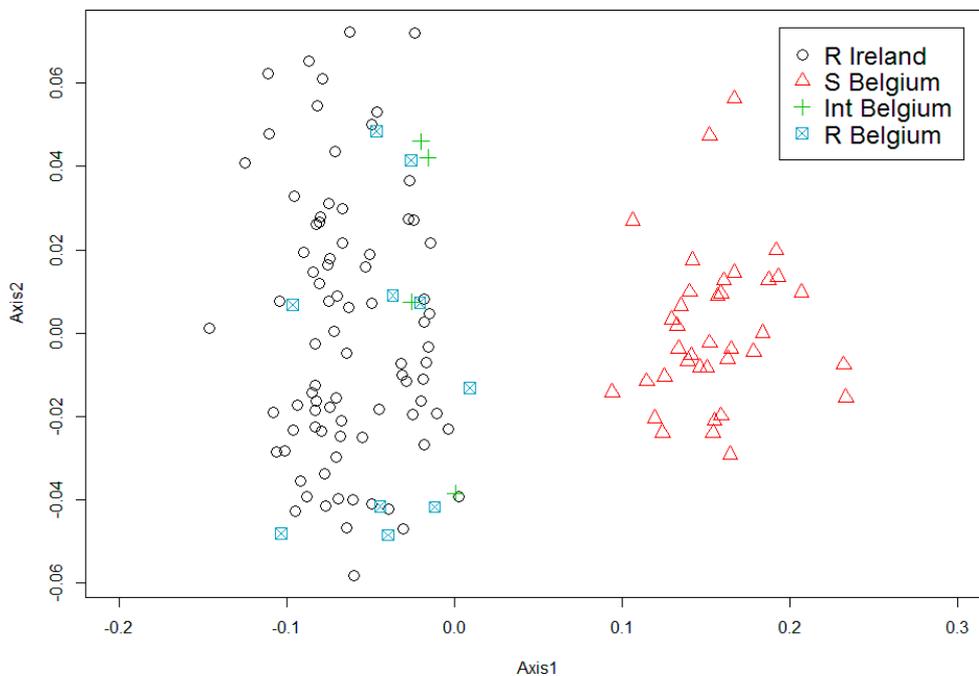


Figure 3. A principal component analysis (PCA) of the ratio between shell measurements for *Corbicula* form R from all Irish sites and forms R, S and individuals with intermediate morphotypes between form R and S (Int), from the River Meuse in Belgium.

3.2. COI Sequence

A 710 bp length fragment of the mitochondrial COI was successfully amplified for 24 of the Irish specimens (Table 2). All the COI sequences retrieved were identical to FW5, the mtCOI haplotype of form A/R distributed in Europe and Americas. All individuals sampled from the Irish populations possessed the same haplotype. No haplotypes from other invasive lineages (Figure 4) were detected from the Irish sites.

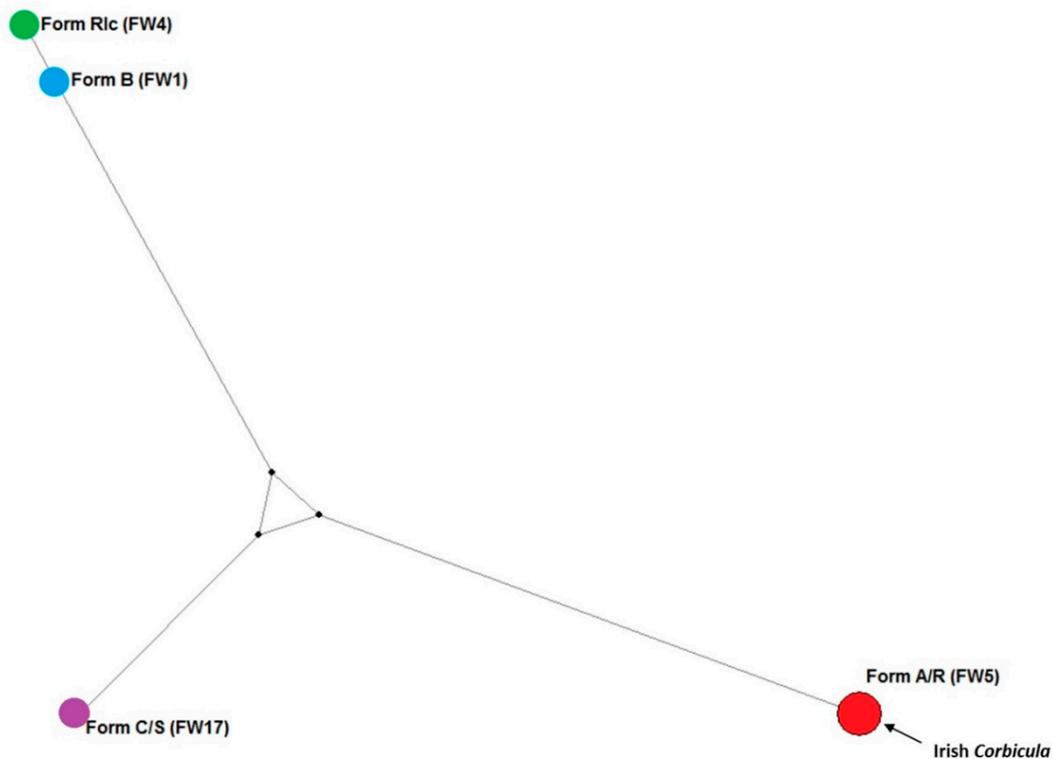


Figure 4. Haplotypic diversity and relationships in Invasive *Corbicula* clams inferred through median-joining Network. The European invasive haplotypes FW1 (Form B), FW4 (Form Rlc), FW17 (Form C/S), FW5 (Form A/R; Irish individuals) are plotted. Branch length was proportional to the number of mutations between the haplotypes and nodes proportional to the haplotype frequency.

3.3. Microsatellite Markers

Genetic diversity and genetic relationships in invasive *Corbicula* clams estimated through a Discriminant Analysis of Principal Components (Figure 5) based on multilocus genotypes from microsatellite marker amplifications show a common clustering between all Irish samples and the American and European invasive form A/R, suggesting that Irish *Corbicula* belongs to this same lineage.

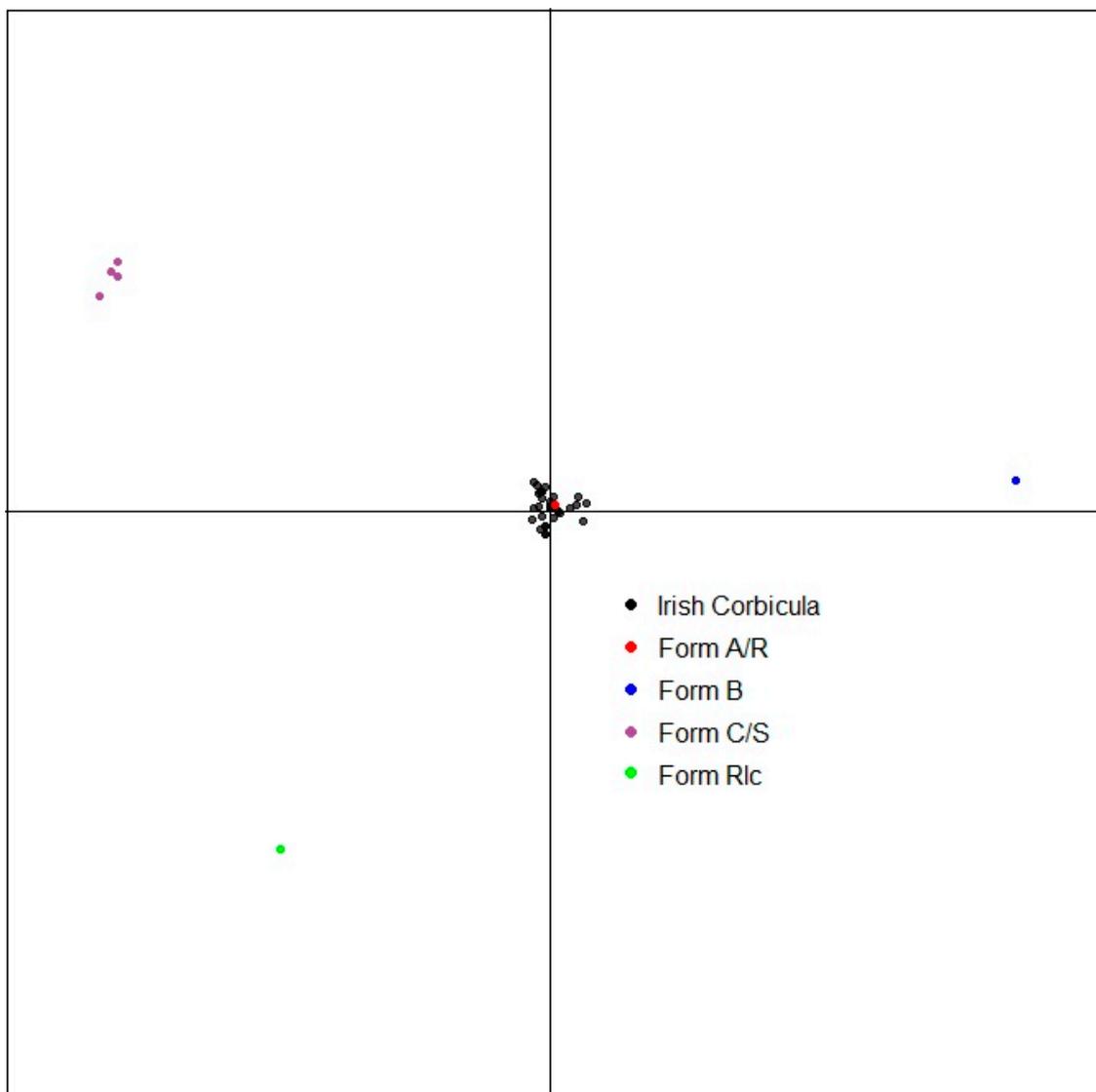


Figure 5. Genetic diversity and genetic relationships in invasive *Corbicula* clams estimated through a Discriminant Analysis of Principal Components based on multilocus genotypes. Only axes 1 and 2 are represented. One dot represents one distinct multilocus genotype; individuals showing the exact same genotype are, therefore, pictured under the same dot.

4. Discussion

Here, we used a multi-method approach, including mitochondrial COI sequencing, microsatellite genotyping and morphological analysis, to identify the invasive *Corbicula* lineages in Ireland. Such an integrative approach was necessary as the genus *Corbicula* is characterized by haplotypes capable of displaying highly divergent phenotypes in response to differing environmental conditions or following cross-lineages mixing.

Our results showed that only the invasive form A/R, the most widespread invasive lineage, was present from all samples collected in Irish waters, as determined using a combination of (a) morphological analysis, (b) mtDNA (COI) and (c) microsatellite markers.

No clams with either a narrow and fine-ridged shell or deep purple interior, as corresponding to form S [19,40] were observed. The PCA analysis carried out on the ratio between shell length and shell height (Figure 3) supports the correct classification of these individuals as belonging to form R.

Morphologically, all clams sampled from the Irish sites conform to the invasive form A/R as described in [11,21,23,28,29], with a certain variability observed (Figure 3).

All individuals sampled from the Irish sites presented the invasive FW5 mtCOI haplotype of form A/R distributed in Europe and Americas [11,21,23,28,29]. The COI sequence data supports the results from the morphological analysis: no mismatches were observed between mtCOI and morphotype, which may be potentially caused by reproduction through androgenesis between distinct lineages [28].

All Irish sampled individuals showed a common clustering for the microsatellite data with the A/R lineage. This form has proven to be clonal and, thus, shows no genetic diversity. Indeed hundreds of individuals of this lineage, sampled at different locations from across Europe, North and South America, present the exact same multilocus genotype with no genetic diversity [11]. The genetic variability detected within Irish populations is very likely due to the poor amplification of the microsatellites (Supplementary Material S2). Initial storage conditions of some samples in methanol was likely responsible for this. It is also a possible that false positives were detected from the data.

It is unlikely that other forms of *Corbicula* exist in Ireland as extensive investigations have not revealed clams displaying differing morphological characteristics [14,16,17] (Minchin Pers Ob.) or in subsequently discovered populations [41,42]. As the level of genetic diversity among the invasive lineages of *Corbicula* clams (reviewed in [18]) was low, it was not possible to discriminate the origin of the Irish populations. The emerging field of massive parallel sequencing (MPS) to detect single nucleotide polymorphisms (SNPs) [43] could potentially complement the approaches used in our study. Similarly, it is not possible to draw any inferences on the number of discrete introductions that may have occurred [44].

The combined methodology of mitochondrial COI sequencing, microsatellite genotyping and morphological analysis has the potential to pick apart the exact identification of genetically complex species groups for which traditional ecological methods may have overlooked complex relationships such as in *Corbicula* [27] with its highly invasive clonal A/R form. The ability to discriminate between discrete introductions to a geographic area, and secondary spread is an invaluable tool in prioritising AIS biosecurity resources [45] and can inform horizon scanning [46].

5. Conclusions

The use of a combined morphological and nuclear marker approach in resolving the identity of the invasive *Corbicula*, demonstrates that only one invasive lineage of *Corbicula* has invaded Ireland, the most prevalent and widely distributed form being A/R, a form also found across Europe and America. The extremely low genetic diversity found within this invasive lineage makes the determination of differences in population origins difficult as a result, and therefore, the number of discrete introductions of *Corbicula* to Ireland remains unknown. In order to properly inform management plans and limit impacts to ecosystem services, the identity of invasive *Corbicula* populations must be resolved. The use of a combined morphological, mitochondrial and nuclear marker approach in gleaning the identity of invasive *Corbicula*, as demonstrated in this study, provides a useful tool for achieving these goals, with the possibility for extending this approach to other invasive species.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/11/8/1652/s1>, supplementary S1, supplementary S2.

Author Contributions: Conceptualization, R.S., E.E. and F.L.; Methodology, R.S., E.E., F.L., K.V.D. and D.M.; Software, R.S. and E.E.; Validation, R.S. and E.E.; Formal analysis, R.S. and E.E.; Investigation, R.S. and E.E.; Resources, F.L. and K.V.D.; Data Curation, R.S., E.E., F.L. and K.V.D.; Writing—Original Draft Preparation, R.S. and E.E.; Writing—Review and Editing, R.S., E.E., F.L., D.M. and K.V.D.; Visualization, R.S. and E.E.; Supervision, F.L. and K.V.D.; Project Administration, R.S., E.E., F.L. and K.V.D.; Funding Acquisition, F.L. and K.V.D.

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

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