Cyfoeth Naturiol Cymru
Natural Resources Wales

Monitoring Allis and Twaite Shad: quality assurance and species identification using molecular techniques

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NRW Evidence Report No. 1

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We work to support Wales' economy by enabling the sustainable use of natural resources to support jobs and enterprise. We help businesses and developers to understand and consider environmental limits when they make important decisions.

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1. Crynodeb Gweithredol

Yn gyffredinol, mae *Alosa* spp. (gwangod) yn bysgod esgynnol sy'n perthyn i'r teulu Clupeidae. Mae'r poblogaethau o herlod (*Alosa alosa* Lacépède) a gwangod (*Alosa fallax* L.) wedi gostwng ar draws eu dosbarthiad daearyddol yn ystod y ganrif ddiwethaf ac er eu bod yn gyffredin o gwmpas arfordir yr Ynysoedd Prydeinig, mae'r poblogaethau silio yn y DU yn gyfyngedig i raddau helaeth i Afonydd Hafren, Gwy, Wysg a Thywi sy'n llifo i Fôr Hafren. O ganlyniad, gwarchodir y ddwy rywogaeth gan ddeddfwriaeth ryngwladol a'r DU. Tra ystyrir bod y niferoedd o *A. fallax* yn eithaf sefydlog yn yr afonydd hyn, nid oes cofnodion silio diweddar wedi'u cadarnhau o *A. alosa*.

Mewn ymateb i'r angen am fonitro statws y boblogaeth er mwyn hysbysu amcanion cadwraeth ar gyfer y rhywogaethau hyn, datblygodd Cyngor Cefn Gwlad Cymru ac Asiantaeth yr Amgylchedd Cymru ddull lled feintiol a chost effeithiol o asesu'r potensial recriwtio drwy samplu wyau gwangod. Fodd bynnag, ni all y dull hwn wahaniaethu rhwng y ddwy rywogaeth o *Alosa*, ac mae perygl y gallai wyau rhywogaethau eraill o bysgod gael eu cofnodi trwy gamgymeriad fel wyau gwangod. Nod y prosiect hwn oedd defnyddio technegau genetig i fynd i'r afael â'r gwendidau hyn.

Canolbwyntiodd yr astudiaeth bresennol ar gasglu wyau o 12 safle samplu o'r tair afon yng Nghymru ble mae gwangod yn silio (Gwy, Wysg a Thywi). Yr amcanion oedd (a) pennu p'un ai fod yr wyau a gasglwyd yn perthyn i *Alosa* spp. a (b) pennu'r gyfran o *A. fallax* ac *A. alosa* gan ddefnyddio tri marciwr genetig gwahanol – DNA mitocondriaidd a dau fath ar DNA cnewyllol: nif1-nDNA a chwe locws microloeren.

Cafodd cyfanswm o 226 wy eu genoteipio'n llwyddiannus. Roedd 85% yn perthyn i *Alosa* spp. gan ddangos lefel dda o adnabod wyau gan y tîm casglu. Canfuwyd bod yr wyau nad oeddent yn wyau *Alosa* yn perthyn i bilcod (*Phoxinus phoxinus*) a chochgangod (*Squalius cephalus)*. O ganlyniad, mae ymlediad y silio tybiedig gan wangod ar y Tywi ychydig yn llai na'r hyn a awgrymir gan yr arolygon wyau yn unig. Fodd bynnag, cadarnhawyd mai ymlediad uchaf y silio gan wangod ar y Gwy yw Pont Brynwern, tua 9km ymhellach i fyny'r afon na'r terfyn uchaf hysbys yn Llanfair-ym-Muallt.

Wrth bennu'r cyfrannau o *A. fallax* ac *A. alosa*, mae'r astudiaeth bresennol wedi dangos nad oedd y marcwyr DNA a ddefnyddiwyd yn ddigon rhywogaeth-benodol i gynhyrchu canlyniadau digamsyniol. O'u hystyried yn ochelgar, ac yn seiliedig ar y DNA mitocondriaidd a'r DNA cnewyllol (nif1-nDNA), y gyfran o *A. fallax* yn Afon Gwy yw 70% gydag 1% o *A. alosa* a 29% o groesrywiau. Yn seiliedig ar yr un marcwyr, y gyfran o *A. fallax* yn Afon Wysg yw 54% gyda 46% o groesrywiau ac yn Afon Tywi, canfuwyd bod 28% yn *A. fallax* gyda 72% yn cael eu hadnabod fel croesrywiau. Mae'n debyg y bydd marcwyr DNA ychwanegol yn dangos canran uwch o groesrywiau. Roedd y loci microloeren yn dangos bod y boblogaeth yn y Tywi yn enetig wahanol i'r rhai yn Afon Wysg a Gwy, ond ni chafwyd strwythur poblogaeth. Roedd gan wy sengl o'r Gwy isaf farcwyr *A. alosa* yn unig a dyma'r dystiolaeth ddiweddar gyntaf o silio gan y rhywogaeth hon yn Afon Gwy.

Mae'r astudiaeth wedi dangos ei bod yn bosibl cyfuno gwaith genetig â gwaith maes cyffredinol gan staff anarbenigol sydd wedi derbyn cyfarwyddiadau ac offer addas. Mae'r marcwyr genetig wedi rhoi llawer mwy o hyder yng nghanlyniadau'r cicsamplu a chynyddu cywirdeb y rhain trwy (a) cadarnhau'r math o wyau a (b) taflu goleuni ar strwythur poblogaeth a chyfansoddiad y rhywogaethau *Alosa*.

Er mwyn gwella cywirdeb o ran adnabod rhywogaethau a rhoi mwy o hyder wrth fesur y cyfraddau o groesrywedd, dylai'r gwaith yn y dyfodol ganolbwyntio ar y defnydd o dechnolegau DNA modern er mwyn datblygu cyfres o farcwyr DNA rhywogaeth-benodol.

2. Executive Summary

Alosa spp. (shads) are generally anadromous fishes belonging to the family Clupeidae. Populations of allis shad (*Alosa alosa* Lacépède) and twaite shad (*Alosa fallax* L.) have declined across their geographic distribution over the last century and despite being widespread around the coast of the British Isles, UK spawning populations are largely restricted to the Rivers Severn, Wye, Usk and Tywi which drain into the Bristol Channel. As a result, both species are protected by UK and international legislation. While numbers of *A. fallax* are considered to be quite stable in these, there have been no recent confirmed spawning records of *A. alosa*.

In response to a need to monitor the population status to inform conservation objectives for these species, the Countryside Council for Wales and Environment Agency Wales developed a semi-quantitative and cost-effective method to assess recruitment potential by sampling shad eggs. However, this method is unable to distinguish between the two *Alosa* species, and there is a risk that eggs of other fish species may erroneously be recorded as shad eggs. The aim of this project was to use genetic techniques to address these weaknesses.

The present investigation focussed on the collection of eggs from 12 sampling sites from the three Welsh rivers where shad spawn (Wye, Usk and Tywi). The objectives were to (a) determine whether the collected eggs belonged to *Alosa spp.* and (b) determine the proportion of *A. fallax* and *A. alosa* using three different genetic markers – mitochondrial DNA and two types of nuclear DNA: nif1-nDNA and six microsatellite loci.

A total of 226 eggs were successfully genotyped. 85% belonged to *Alosa* spp. demonstrating that a good level of egg identification in the collection team. Non-*Alosa* eggs were identified as belonging to minnow (*Phoxinus phoxinus*) and chub (*Squalius cephalus*). As a result, the extent of putative shad spawning on the Tywi is slightly less than suggested by the egg surveys alone. However, the upper extent of shad spawning on the Wye was confirmed as being Brynwern Bridge, about 9km further upstream than the previous known upper limit at Builth Wells.

In determining the proportions of *A. fallax* and *A. alosa*, the present study has demonstrated that the DNA markers used were not sufficiently species-specific to provide unequivocal results. Taken with caution, based on the mitochondrial DNA and nuclear DNA (nif1-nDNA); the proportion of *A. fallax* in the Wye is 70 % with 1% *A. alosa* and 29% hybrids. Based on the same markers, the proportion of *A. fallax* in the Usk is 54% with 46% hybrids and in the Tywi, 28% were identified as *A. fallax* with 72% identified as hybrids. Additional DNA markers will likely identify a higher percentage of hybrids. Microsatellite loci indicated that the Tywi population is genetically different from the Usk and the Wye, but no population structure was found. A single egg from the lower Wye had only *A. alosa* markers and is the first recent evidence of spawning of this species in the Wye.

This study has demonstrated that it is possible to integrate genetic work into standard fieldwork by non-specialist staff supplied with suitable instructions and equipment. The genetic markers greatly increased confidence in and resolution of the kick

sampling results by (a) confirming the identity of the eggs and (b) providing insights into population structure and *Alosa* species composition.

In order to enhance the resolution of species identification and increase confidence in quantifying rates of hybridisation, future work should focus on the use of modern DNA technologies to develop a suite of species specific DNA markers.

3. Introduction

3.1. Background

The Allis shad (*Alosa alosa*) and the twaite shad (*Alosa fallax*) are anadromous fish species which belong to the Clupeidae (herring family) (Maitland & Hatton-Ellis, 2003). As a result of river management schemes that block spawning migration routes through inhibiting the longitudinal connectivity of their native rivers, their populations are threatened and have indeed collapsed in some rivers (Aprahamian *et al*. 1999; 2002). Due to declining populations, both species are protected under the EU Habitat and Species Directive (Annexes II and V) and the Bern Convention (Appendix III). In the UK, *Alosa* spp. are also protected under the Wildlife and Countryside Act (1981) and are Priority Species in the UK Biodiversity Action Plan.

In Wales, all three rivers in which spawning populations of *Alosa* spp. are still present (Aprahamian *et al*. 1999) have protection as Special Areas of Conservation (SACs). These are the Rivers Wye, Usk and Tywi (Towy). Within these sites there is a need to monitor the status of shad populations so that appropriate management measures can be taken, and in support of the UK's international reporting requirements to the European Commission.

Due to the complex life history of anadromous *Alosa* spp., and in particular, the limited duration that larvae reside at natal sites, monitoring temporal and spatial trends in population recruitment success using standard sampling methods presents a considerable challenge. Various monitoring methods have been attempted including hydroacoustic monitoring of adult migration and seine netting of juvenile life stages, but these have been both technically difficult, costly and relatively inefficient (Gregory 2000; Noble *et al.* 2007). Although it provides only relatively basic population information, kick sampling of shad eggs (Figure 1) has been shown to be a cost-effective method to semi-quantitatively gather information on spawning activity and distribution (Thomas & Dyson 2012a, 2012b).

Figure 1: Adult twaite shad (left) and shad egg sampled using kick sampling (right). Adult shad reproduced by kind permission of the Wye & Usk Foundation. Egg photo © NRW.

The use of eggs has proved useful as a monitoring tool. However, there is considerable morphological and meristic similarity between the adult life stages of the two *Alosa* spp. found in the British Isles (Aprahamian *et al*. 1999; 2002) and despite some reported variation in the range of egg diameter, no reliable diagnostic criteria are currently available to distinguish the eggs of *Alosa* spp (Aprahamian *et al*. 2002). In addition, hybrids between these two species have also been reported in Welsh and other rivers (Maitland & Hatton-Ellis 2003; Alexandrio & Faria, 2004) which cannot be identified through egg morphology. Surveyors often report that some eggs also possibly belong to non-target species that further complicate population estimation. Thus, egg morphology only has limited utility for *Alosa* spp. monitoring. However, molecular tools are increasingly used to identify problematic species for which morphology may be difficult to identify to species level.

3.2. Project Objectives

Accordingly, the key objectives of the current study were to:

- Test the feasibility of collecting egg samples suitable for genetic analysis using Natural Resources Wales (NRW) staff supplied with suitable equipment and instructions.
- Quality assure the eggs collected by the kick sampling procedure in order to confirm that they are shad eggs.
- Explore and develop the potential to apply low-cost genetic techniques that can be used to identify the eggs of *A. alosa, A. fallax* and their hybrids.

4. Egg Collection and Preparation

4.1. Sampling

During the spawning season (June 2013), NRW staff collected 240 individual eggs by kick sampling from 12 sampling sites in the rivers Tywi, Usk and Wye (Figures 2 & 3) using a previously used protocol (see Thomas & Dyson 2012a for details). Staff were issued with clear instructions (Appendix A) and standard field equipment including pre-labelled 1.5ml Eppendorf tubes pre-loaded with buffer solution and coolboxes. Up to 20 eggs were collected from each sampling site and placed in ATL (lysing) buffer in pre-labelled 1.5ml Eppendorf tubes (Table 1). Eggs were either immediately couriered by overnight service to the laboratory, or else refrigerated overnight and couriered the following day.

Sample acquisition was generally successful, except for the premature hatching of some of the eggs collected from one site on the River Tywi (T3). This occurred when the samples were fixed in the ATL buffer (Qiagen) and is considered to be due to the embryos experiencing stress when already in an advanced stage of development. While hatching is unlikely to occur during earlier stages of embryological development, the separation of free embryos from the egg has not impacted on the success of the analytical techniques used during the current study.

River	NGR	Site Name	Site Code	Sampling Date	Number of eggs fixed in ATL
Wye	SO011568	Brynwern Bridge	W1	10/06/2013	20
	SO034515	Afon Irfon, Gro Park	W2	4/06/2013	20
	SO159379	Spread Eagle	W3	4/06/2013	20
	SO234443	Boatside	W4	4/06/2013	20
	SO511125	Monmouth	X1	13/06/2013	20
Usk	SO374005	Usk Town	U1	5/06/2013	20
	ST386969	Llantrisant	U2	5/06/2013	20
	SO367024	Prioress Mill	U3	6/06/2013	20
	SO296137	Castle Meadows, Abergavenny	U4	6/06/2013	20
Tywi	SN493203	Nantgaredig Bridge	T1	11/06/2013	20
	SN467214	White Mill	T2	11/06/2013	20
	SN500220	Cothi Confluence	T3	13/06/2013	20

Table 1: Sample list of the egg samples received during 2013

Figure 2: Spawning grounds on the River Usk at Abergavenny (Site U4). Photograph © NRW.

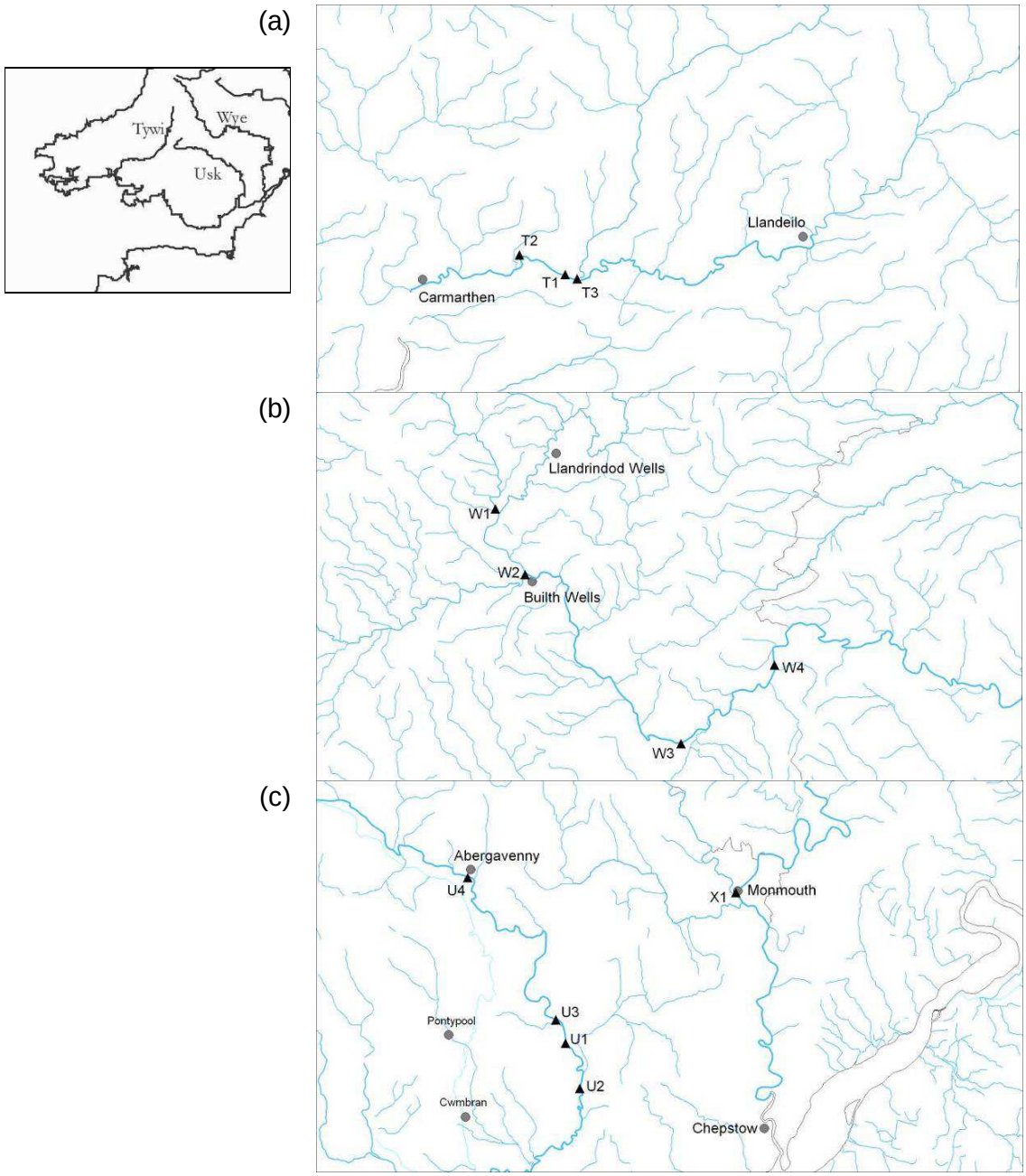

Figure 3**:** Inset: map of South Wales showing the three rivers (Tywi, Usk and Wye). (a) Location of Tywi sampling sites; (b) Location of Upper Wye sampling sites; (c) Location of Usk and Lower Wye sampling sites. © Crown Copyright and database right 2013. Ordnance Survey 100019741.

4.2. Laboratory Preparation

The DNA was extracted in batches of 17-20 eggs. For every batch a negative DNA extraction (i.e. without biological material) was performed to ensure the non contamination of the reagents. In order to ensure the good quality of the DNA and to detect its potential degradation, the extracted DNA was electrophoresed through a 1% agarose gel.

Out of the 250 DNA extractions performed (including negative controls), 226 eggs were successfully extracted. All extraction negatives did not show any DNA, indicating that there has been no cross contamination during the extraction process. The DNA concentration of all samples was determined using a fluorospectrometer (Thermo Scientific). Some samples showed very low DNA concentration (Appendix B for DNA quantities of all samples). Overall, DNA quality and concentration were satisfactory for all sampling locations.

5. Distinguishing Shad and Non-shad Eggs

Two mitochondrial (mtDNA) haplogroups for the cytochrome B (CytB) gene have been previously identified in *Alosa* spp.; haplogroup A and haplogroup F are mainly associated with *A. alosa* and *A. fallax* respectively (Alexandrino *et al*. 2006; Faria *et al*. 2011).

Using the primers described in Alexandrino *et al*. (2006), a fragment of 401 bp of the CytB was successfully amplified for most eggs. Out of the 226 eggs extracted, 191 samples were amplified using CytB specific primers. All extraction negative and PCR negative controls have shown no amplification indicating that there has been no contamination. For the sampling location T1 of the Tywi River, all extracted eggs showed high DNA concentrations and good DNA quality but no amplification with the CytB specific primers. This suggests that the eggs collected belong to non-*Alosa* species. Similar results were also obtained for U3 in the Usk. In this sampling location, 2 of the 17 extracted eggs have shown amplification with the *Alosa* spp. specific CytB primers. All non-shad eggs were checked with universal primers in order to define species identification and were excluded from the mtDNA analysis (section 4).

One of the objectives of this project was to provide NRW with a rapid and cost-effective tool to identify unclassified eggs. Distinguishing the eggs of shad from other freshwater fishes using morphological characters alone can be challenging. The method of choice was DNA barcoding where a short genetic marker, usually the COI gene, of an unknown organism was sequenced using universal primers, to identify to which species it belongs. This method kept analysis costs at a minimum as only one additional PCR and sequencing reaction is required to test the presence of several fish species. Several primer cocktails exist for fish species and it was decided to use the cocktail primers C_FishF1t1 – C_FishR1t1 from Ivanova *et al*. (2007) as this has demonstrated an average sequencing success of 95.2% when tested with 94 fish families (Ivanova *et al*. 2007).

5.1. Methodology:

A segment of the 401 nucleotides of the mitochondrial CytB gene was amplified using the primers from Alexandrino *et al*. (2006).

PCR conditions were:
Buffer: 1x,
MgCl2: 2mM,
dNTPS: 0.2mM,
Primers (forward and reverse): 0.25 µM
Taq: 0.5Unit (Promega Flexi Taq) per sample
DNA concentration: 50-100 ng
Total volume: 32 µl.

PCR Cycling conditions were:
2 min at 94°C
94°C for 45s
60°C for 45s } 35 cycles
72°C for 45s
72°C for 2 min.

A sequence of 553 bp of COI was amplified using the conditions below:

Technical specification of PCR reaction condition:
Buffer: 1x,
MgCl2: 2mM,
dNTPS: 0.2mM,
Primers (for each) 0.25 µM
Taq: 0.5Unit (Promega Flexi Taq) per sample
DNA concentration: 50-100 ng

The PCR Cycling conditions were:
2 min at 94°C
94°C for 30s
48°C for 40s } 35 cycles
72°C for 60s
72°C for 10 min.

5.2. Results and Conclusions:

The COI gene was amplified for all potential non-shad eggs except for the three samples from the River Tywi (T3_01, T3_14 and T3_16). These samples had very low DNA concentrations (below 2 ng) which could explain the lack of amplification. Two different haplotypes were found for the COI gene in the tested individuals. One corresponded to the population T1 and the other to the population U3 and the individual W2_14.

Species corresponding to these haplotypes were assigned to potential species present in the different rivers using sequences from the online DNA sequence database search tool BLAST and plotting the data as a neighbour-joining tree using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2007). Results confirmed that the eggs from T1 were minnow (*Phoxinus phoxinus)* and the eggs from U3 and W2_14 were chub (*Squalius cephalus*) (Figure 4).

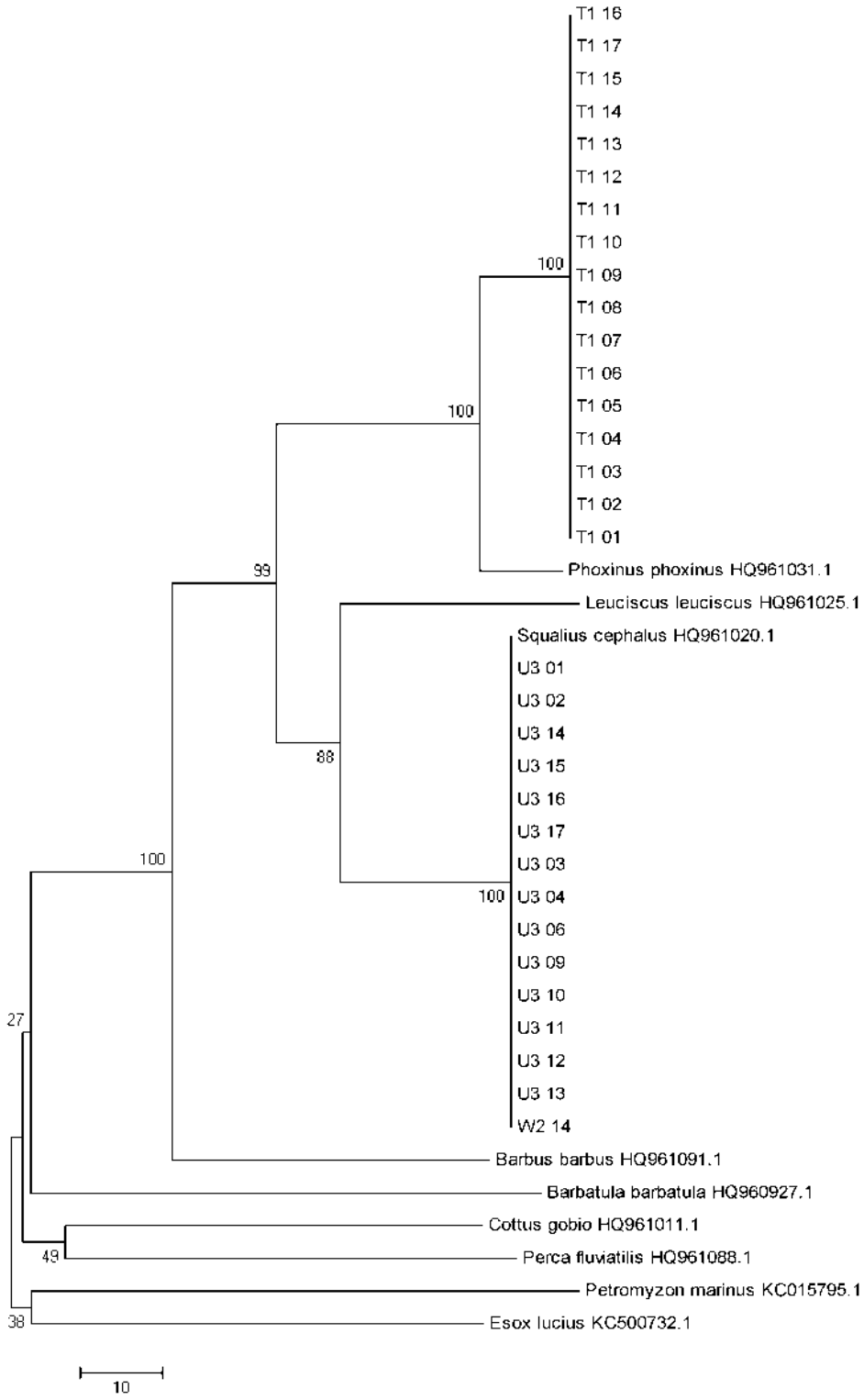

Figure 4: Neighbour-joining consensus tree with 1000 bootstrap replicates drawn using MEGA. Endgroups with format U1 01 are eggs; endgroups with format Barbus barbus AB123456.1 are sequences obtained from BLAST.

Due to overlapping habitat utilisation, it is not unexpected that other lithophilic spawning species are present within the samples of shad eggs. Indeed, spawning activity of one species can often stimulate other species to spawn. This is commonly observed in minnow (*Phoxinus phoxinus*) which often opportunistically capitalise on the disturbance and cleaning of gravels by larger species (A. Pinder *pers. com.*). Accordingly, where the presence of other species can not be confirmed using egg size and embryonic identification, then genetic typing should be used to confirm the species identity of eggs. It should be noted that at site U3, the surveyors were uncertain of the identification of some of the eggs, noting that '8-18 are slightly smaller, slightly yellower'. Other surveyors did not record any observed difference between shad and non-shad eggs.

6. Mitochondrial DNA to Identify *Alosa* spp.

Two mitochondrial (mtDNA) haplogroups for the cytochrome B (CytB) gene have been previously identified in *Alosa* spp.; haplogroup A and haplogroup F are mainly associated with *A. alosa* and *A. fallax* respectively (Alexandrino *et al*. 2006; Faria *et al*. 2011).

6.1. Methods:

Using the primers described in Alexandrino *et al*. (2006), a fragment of 401 bp of the CytB was successfully amplified for most eggs (see section 3).

All PCR amplified products were electrophoresed through an agarose gel in order to confirm a successful amplification with the correct size product. Samples which showed amplification of the expected fragment size were subsequently sent for Sanger sequencing at Beckman Genomics. Sequences were then cleaned and aligned using Codon Code Aligner (CodonCode Corp.), Mega (Tamura *et al*. 2007) and BioEdit (Hall 1999), to determine the *Alosa* haplotypes present.

6.2. Results and Conclusions:

A sequence of 401 bp for 191 individuals was successfully obtained. A copy of the alignment from these sequences as well as the reference shad haplotypes from Alexandrino *et al*. (2006) has been provided as an electronic fasta file format (see data archive appendix for details). Three distinct *A. alosa* haplotypes were detected and five *A. fallax* haplotypes (Table 2). Two non-described haplotypes were identified: "new *fallax*" is present in 3 individuals from the Tywi River (T2 and T3) and "new *fallax* 2" only found in one individual in the River Usk (U4). These two haplotypes differed from the common haplotype Af2 by only a single nucleotide.

		***"A. alosa"* Haplotype**			***"A. fallax"* Haplotype**				
Site	N	A3	Aa1	Aa3	Af1	Af2	Af3	new fallax	new fallax2
W1	20					100.0			
W2	19		26.3	5.3		57.9	10.5		
W3	20		15.0	5.0		65.0	15.0		
W4	19		15.8		21.1	63.2			
X1	20		20.0	5.0		70.0	5.0		
U1	20	5.0	10.0	20.0	5.0	60.0			
U2	20	5.0	30.0	20.0	5.0	40.0			
U3	2					100.0			
U4	18		11.1	22.2	5.6	55.6			5.6
T2	20	50.0	30.0			15.0		5.0	
T3	13	23.1	23.1	7.7		30.8		15.4	

Table 2: Proportion of the different mitochondrial haplotypes per sampling site. *Alosa alosa* are represented by haplotypes A3, Aa1, Aa3 and *A. fallax* are represented by Af1, Af2, Af3, new *fallax* and new *fallax*2.

Across all populations, the percentage of eggs assigned to *A. alosa* was 34 % whereas the percentage of eggs assigned to *A. fallax* was 66 %. The proportion of both species haplotype varied between rivers with respective *A. alosa* : *A. fallax* ratios of approximately 1 : 5 in the Wye, 2 : 3 in the Usk and 7 : 3 in the Tywi (Table 2). Interestingly, these results are in accordance with the previous study from Alexandrino & Faria (2004) which described an increase in the percentage of *A. alosa* haplotypes from the east to the west of Wales, with the group Wye-Teme-Severn having approximately 20-26% *A. alosa*, Usk 49% and Tywi populations 71% (Alexandrino & Faria, 2004). Results from the current study therefore suggest that the proportion of *A. alosa* and *A. fallax* mitochondrial haplotypes has remained stable between 2004 and 2013 in these three rivers.

Alexandrino & Faria (2004) identified the two shad species using morphological characters, they then compared the morphological identification to the CytB gene one. Their morphological analysis did not identify any *A. alosa*, however, the genetic data based on the CytB gene identified 37.8% of their samples as "*A. alosa*" haplotype. This result can only be explained by present or past hybridization between *A. alosa* female(s) and *A. fallax* male(s) (Alexandrino & Faria, 2004). The percentage occurrence of both species haplotypes are similar to the ones identified during the present study.

In combination, the results presented in this report and in Alexandrino & Faria (2004), indicate that mtDNA (CytB gene) cannot be used to differentiate between *A. alosa* and *A. fallax* in the Welsh populations due to widespread hybridization, especially in the Tywi.

7. Using Nuclear Regions and Restriction Fragment Length Polymorphism (RFLP) to distinguish *Alosa* spp

As demonstrated in Section 4, it is difficult to distinguish between *A. alosa* and *A. fallax* species using only mitochondrial DNA. Faria *et al*. (2011) described two Single Nucleotide Polymorphisms (SNPs) at the sequence positions 87 and 88 of the nif1-nDNA sequence (Figure 5). Interestingly, the allele nif1-nDNA*1 (C at position 87) was shown to be the most frequent in *A. alosa* populations, while allele nif1-nDNA*2 (G at position 87), the most frequent in *A. fallax* populations. Accordingly, these could potentially provide species markers (in 90% of the cases). Faria *et al*. (2011) investigated this polymorphism using a SSCP method. However, this method is time consuming and results can potentially be difficult to analyse. The approach taken for the current study has been to develop a RFLP method to investigate this polymorphism. The RFLP technique provides a rapid, cost effective and reliable method to determine genotype using a simple PCR and a restriction enzyme. The two SNPs at sequence position 87 and 88 occur in the recognition site of a restriction enzyme: HaeIII (Figure 5) which have been used here.

Figure 5: Alignment of nif1-DNA from *A. fallax* and *A. alosa* (from Faria *et al*. 2011). The blue rectangle shows the motif recognition of the restriction enzyme HaeIII.

7.1. Methodology:

In order to develop the method, ninf1-nDNA was sequenced using primers described in Faria *et al*. (2011) for a sub-sample of 15 eggs to ensure the presence of the polymorphic sites in Welsh populations. An electronic copy of the alignment has been provided in an electronic fasta file format as an appendix to the present report. Once the presence of the polymorphism was confirmed, all shad individuals were screened at the ninf1-nDNA locus.

Technical specification of the PCR condition:
Buffer: 1x,
MgCl2: 2mM,
dNTPS: 0.2mM,
Primers (forward and reverse) 0.4 µM
Taq: 0.5Unit (Promega Flexi Taq) per sample
DNA concentration: 50-100 ng
Total volume: 12 µl.

The PCR Cycling conditions were:
2 min at 94°C
94°C for 30s
54°C for 30s } 40 cycles
72°C for 30s
72°C for 2 min.

Restriction enzyme digestion:
Template PCR 12µl
Buffer x1
HaeIII 10U
Total volume: 50µl

Enzymatic reaction: 1 hour at 37°C followed by 20 min at 80°C to deactivate the enzyme.

The restriction digests were migrated through an agarose gel and scored. As the signal of heterozygote individuals could be due to incomplete enzymatic digestion, all heterozygote individuals were sequenced.

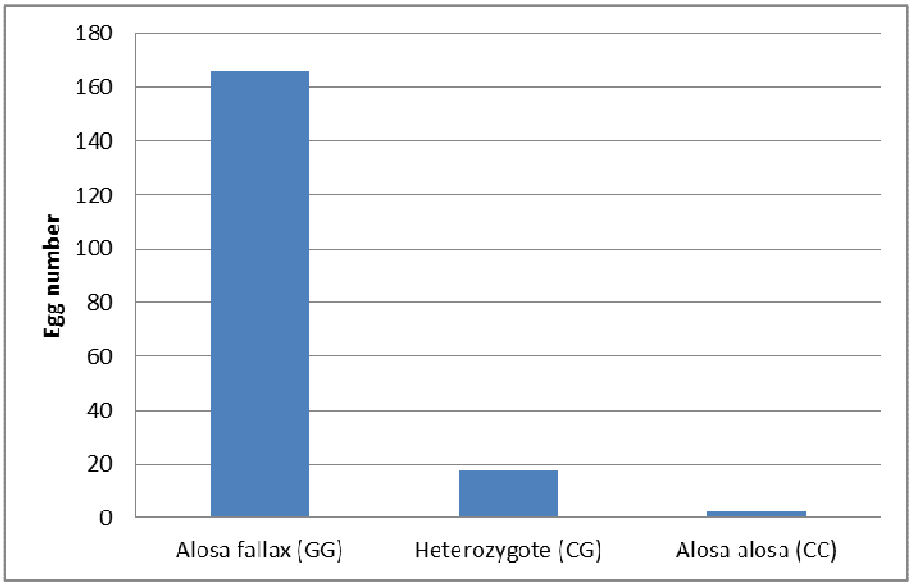

Figure 6: Number of eggs assigned to *A. fallax* and *A. alosa* using the nif1-nDNA region.

7.2. Results and discussion:

Out of the 191 Shad DNA samples tested, 186 successfully amplified the nif1-nDNA region. The majority of samples (166) were homozygote GG which was associated with *A. fallax*; 18 were heterozygote GC and only 2 were homozygote CC (*A. alosa*) (Figure 6). Both homozygote *A. alosa* individuals originated from the X1 sampling site (lower River Wye).

The RFLP method developed here provides a cost-effective and rapid assessment tool to distinguish twaite and allis shad and their hybrids. The heterozygote

individuals were confirmed using the Sanger sequencing method. With the nif1-nDNA region, the majority (89.2%) of the eggs were homozygote for nif1-nDNA*2 (GG) and were therefore associated with *A. fallax*. The presence of heterozygotes (9.7% - GC) could be the result of hybridisation between both species. Only two samples (1.1%) were homozygote (CC) for *A. alosa*.

The observed nif1-nDNA allele frequency difference between the two species has demonstrated high discriminative power compared to the CytB (Faria *et al*. 2011). However, nif1-nDNA was not 100% accurate. Currently, by only using nif1-nDNA and the mitochondrial CytB, the degree of hydridization is underestimated as the only hybrids detectable are the animals displaying markers of both species or the heterozygote for nif1-nDNA (Table 3 and 4), when hybridization can occur at every locus in the genome. Furthermore, as neither of these markers are 100% accurate, the identification of hybrids is even more uncertain.

In order to advance reliable species identification from shad eggs a new species specific marker will be required.

River	*Alosa fallax*	*Alosa alosa*	Hybrids
Wye	70%	1%	29%
Usk	54%	0%	46%
Tywi	28%	0%	72%

Table 3: The proportion of eggs which can be assigned to *A. fallax* and *A. alosa* using both the mitochondrial DNA Cyt B and the nuclear marker nif1-nDNA. The percentage of hybrids, i.e. the proportion of eggs which had opposing species identities based on these markers is also included.

The data presented in Tables 3 and 4 provide a summary of the results for CytB and nuclear marker nif1-nDNA. Combining these two markers has indicated that hybridization is very high in the Tywi (72%), intermediate in the Wye (46%) and lower in the Usk (29% - Tables 3 and 4). Only one egg was found with both *A. alosa* markers (Tables 3 and 4). It is also noteworthy that most hybrids exhibit an *A. fallax* nuclear marker and an *A. alosa* mitochondrial marker. As mitochondria are maternally inherited, this suggests that the hybridizations observed are the progeny of *A. fallax* males and *A. alosa* females.

		CytB								nif1-nDNA		
		A. alosa			*A. fallax*					*A. alosa*	hybrid	*A. fallax*
Site	N	A3	Aa1	Aa3	Af1	Af2	Af3	nf	nf2	nif1-nDNA*1	hetero-zygote	nif1-nDNA*2
W1	19	0	0	0	0	19	0	0	0	0	4	15
W2	20*	0	5	1	0	11	2	0	0	0	0	20
W3	20	0	3	1	0	13	3	0	0	0	0	20
W4	19	0	3	0	4	12	0	0	0	0	7	12
X1	20	0	4	1	0	14	1	0	0	2	1	17
U1	20	1	2	4	1	12	0	0	0	0	1	19
U2	20†	1	6	4	1	8	0	0	0	0	2	16
U3	2†	0	0	0	0	2	0	0	0	0	0	1
U4	19*	0	2	4	1	10	0	0	1	0	1	18
T2	20†	10	6	0	0	3	0	1	0	0	1	16
T3	13	3	3	1	0	4	0	2	0	0	1	12

Table 4: Number of eggs which can be assigned to *A. fallax* and *A. alosa* using both the mitochondrial DNA Cyt B and the nuclear marker nif1-nDNA. * One or more Cyt-b samples did not amplify; † One or more mitochondrial samples did not amplify.

8. Use of Microsatellites to assess Population Structure

Using the genetic markers – CytB and nif1-nDNA – the frequency of hybridization between *A. alosa* and *A. fallax* was underestimated as hybrid detection was limited to those individuals exhibiting contrasting species results for each marker. To improve hybrid identification, the revised approach utilised the seven microsatellite loci described for *A. alosa* and *A. fallax* by Faria *et al*. (2004).

8.1. Methodology:

Amplification conditions were optimised using the DNA confirmed as *Alosa* sp. with both CytB and nif1-nDNA.

Technical specification of the PCR conditions:
Buffer: 1x,
MgCl2: 2mM,
dNTPS: 0.2mM,
Primers (forward and reverse) 0.8 µM
Taq: 0.5Unit (Promega Flexi Taq) per sample
DNA concentration: 20 ng
Total volume: 12 µl.

The PCR Cycling conditions were:
3 min at 94°C
94°C for 30s
*°C for 30s } 35 cycles
72°C for 30s
72°C for 3 min.

*: Loci AF13, AA14 and AA20 - 59 °C; ASA9, ASA2 and ASA8 – 50 °C; AF20 - 58 °C.

All *Alosa* sp. were amplified for each of the 7 microsatellite loci. Following amplification, the PCR template were pooled together in order to reduce the genotyping cost. Locus AF13 was pooled with locus AA14; Locus AF20 was pooled with AA20 and Locus ASA9 was pooled with ASA2. ASA8 was left as a single reaction. Prior to genotyping all reactions (pooled and single) were diluted by a factor of 1/600.

Only microsatellites with at least 60% of the individual genotyped were used, therefore Asa9 was discarded from the analysis. Only individuals with three or more microsatellites genotyped were used in the analysis.

	N	H_{exp}	H_{obs}	Average number of alleles per locus
T2	18	0.49	0.52	3.50
T3	13	0.43	0.44	3.17
U1	13	0.46	0.52	2.83
U2	16	0.50	0.52	3.83
U3	1	0.10	0.20	1.20
U4	15	0.44	0.49	3.33
W1	18	0.45	0.49	3.33
W2	19	0.47	0.41	4.67
W3	19	0.46	0.38	3.67
W4	19	0.44	0.49	3.33
X1	17	0.43	0.52	3.00

Table 5: Expected (H_{exp}) and Observed (H_{obs}) Heterozygosity and average number of alleles per locus in the different sampling sites.

8.2. Results and Conclusions:

Genotypes were successfully obtained for 168 individuals from the three rivers (Table 5). Genetic diversity was observed to be low with an average 3.5 allele per locus (U3 was excluded due to the low sample size). Observed heterozygosity was similar between all populations (except for U3). To analyse the population genetic structure of Welsh populations, F_{IS} and F_{ST} values representing the inbreeding coefficient and the population differentiation respectively were calculated using Genetix (Belkhir *et al*. 2004 - Table 6). F_{ST} values were low suggesting that the microsatellite makers used did not have sufficient power to differentiate between the Tywi, Usk and Wye. AF13 represents the locus with the most discriminant power. F_{IS} was low for most of the loci except AF20 and AA14.

Marker	F_{IS}	F_{ST}
AA20	-0.10479	0.04929
AF20	0.39765	0.08249
ASA8	-0.03222	0.02769
AA14	0.35196	0.02477
AF13	-0.10395	0.13068
Asa2	-0.10055	0.07022

Table 6: Table of F_{ST}, F_{IS} and F_{IT} per locus.

Correspondence Analysis (CA) taking the centroids of each population as active elements using the AFC-3D procedure of Genetix 5.04 (Belkhir *et al*. 2004) was performed (Figure 7). The three first axis explained 52.35% of the total inertia. Interestingly, there is a geographical component on Axis 1 (28.35%) with from left to right, the Wye, the Usk and the Tywi. The Tywi population appeared to pull the signal of Axis 1. Axis 2 (15.29%) and Axis 3 (13.21%) are pulled by population U3. This is most probably an artefact of the small sample size of U3. In order to infer the genic difference between the three rivers, an exact test for genic differentiation was performed using Genepop (Rousset 2008 – Table 7). The exact test is concerned with the distribution of alleles for the various rivers. The null hypothesis is that alleles are drawn from the same distribution in all populations. Clearly, the null hypothesis can be rejected when the Tywi population is compared to the Usk and the Wye. The p-value is also significant between the Usk and the Wye.

	Tywi	Usk
Usk	0.000	-
Wye	Highly sign.	0.048

Table 7: Genic differentiation between pairs of populations calculated using exact tests with the program Genepop. The p-values are presented in the table.

In order to infer population structure, the software STRUCTURE (Pritchard *et al*. 2000) was used. In this analysis, the software uses the microsatellite dataset and divide it in a number K of populations. The parameters for this analysis were as follow: 200 000 burning followed by 500 000 MCMC with the admixture model for a K equal 1 to 3. Each simulation was run five independent times for each value of K. The results are presented in Figure 8. Interestingly, these data do not display any structure (Figure 8), which suggest that the samples obtained from the three rivers can be considered as a single population. This result is unexpected as the CA and the exact test on genic differentiation indicated differentiation of the Tywi when compared to the Usk and the Wye. Increasing the number of microsatellite loci should assist in resolving this ambiguity.

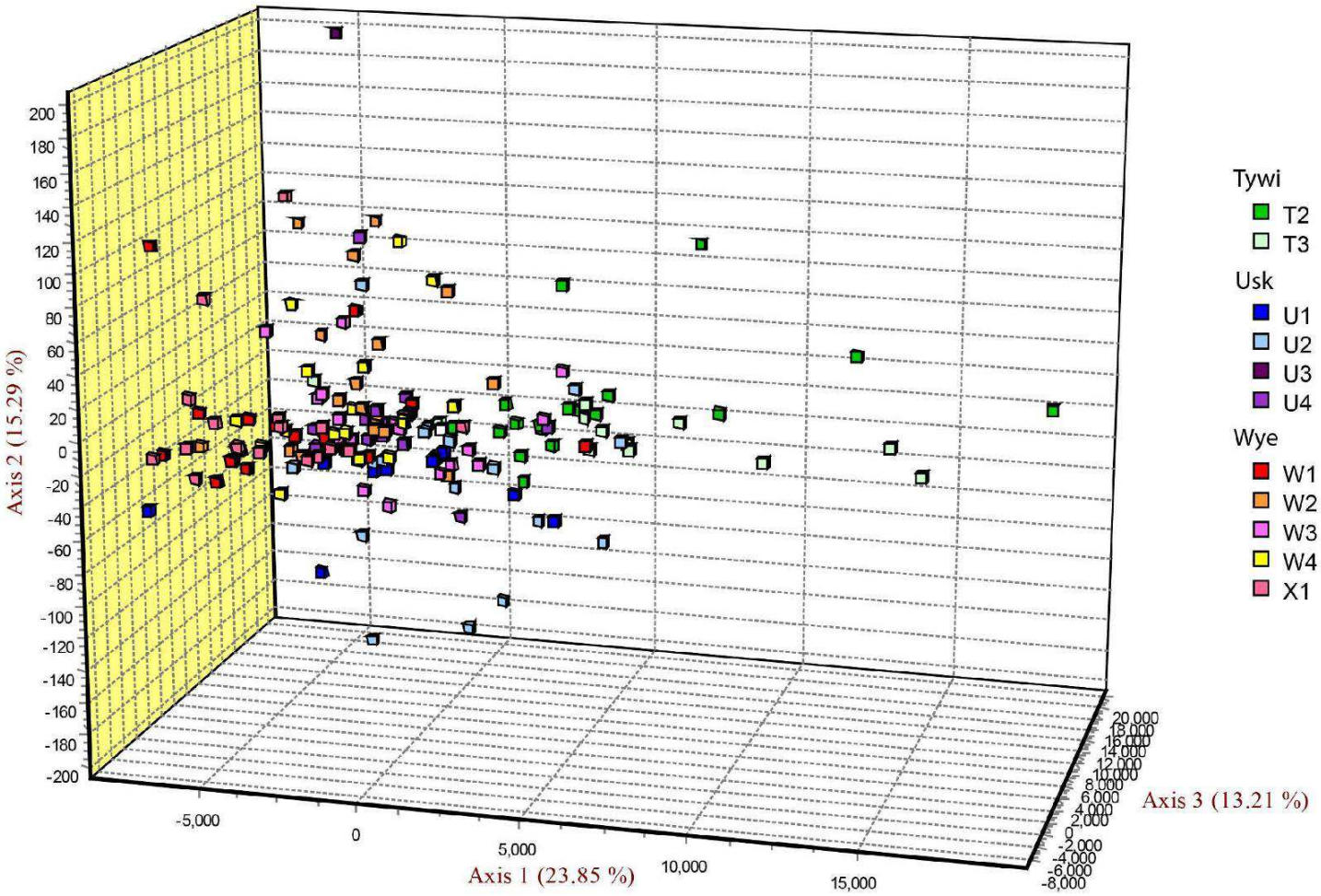

Figure 7: CA plot with three axes displayed. Every square represents an individual.

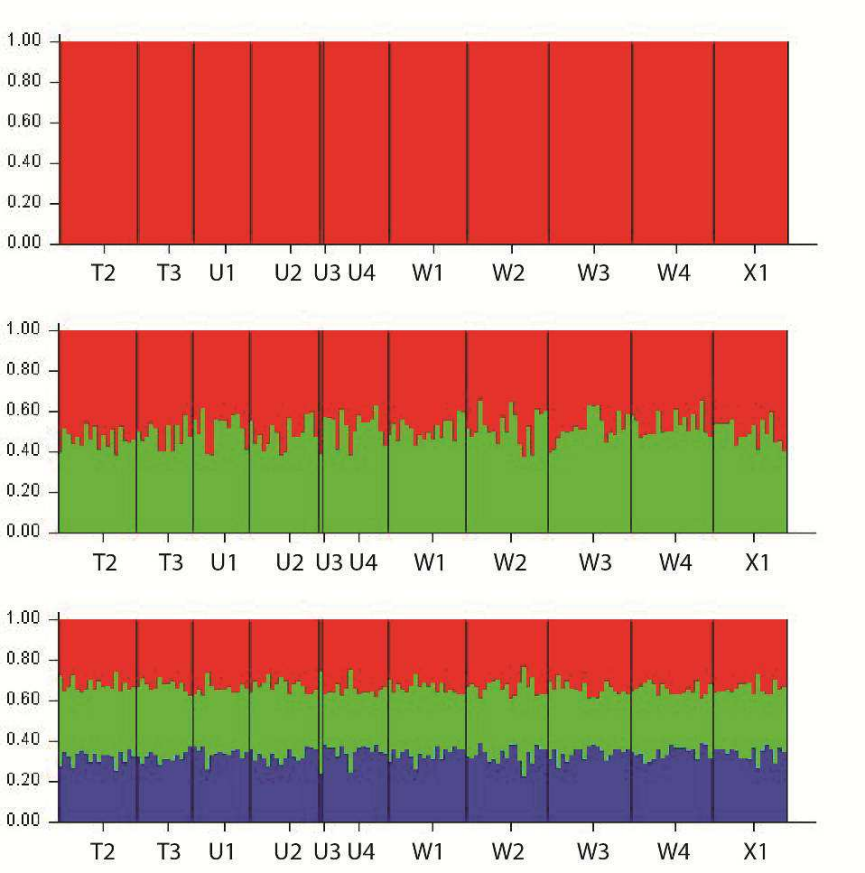

Figure 8: Example of the Structure results from K=1 to K=3. The different individuals are presenting in the form of a column and the population names of the samples can be read below each of the diagram.

9. Conclusions and possible future work

9.1. Conclusion

The project objectives were to use genetic markers to survey *A. alosa* and *A. fallax* populations - and their hybrids - using the eggs collected from three Welsh rivers, in order to (i) test the feasibility of using NRW staff to collect genetic material (ii) provide a quality assurance method for assessing whether the eggs were shad and (iii) identify what species were present. Three types of genetic marker were used: (1) mitochondrial CytB gene, (2) nif1-nDNA gene and (3) microsatellites. The results indicate that:

- NRW staff collecting material for routine monitoring purposes are, when supplied with clear instructions and equipment, well placed to sample good quality genetic material.

- A high proportion (85%) of shad eggs were correctly identified in the field. Based on this level of accuracy, in the absence of genetic analysis, two of the twelve sites surveyed would have been erroneously recorded as supporting the spawning of shad. Whilst this level of taxonomic field accuracy is encouraging, this clearly demonstrates the importance of genetic techniques to validate egg identification to support catchment and species management decision processes.

- The upper limit of spawning on the Wye was confirmed as being Brynwern Bridge, about 9km further upstream of the previous upstream limit.

- Across all populations, the percentage of eggs assigned to *A. alosa* was 34%, whereas the percentage of eggs assigned to *A. fallax* was 66% using the Cyt B. However, this marker is a poor species marker and cannot be used with confidence for species identification in the Welsh shad populations.

- Using the nuclear nif1-nDNA marker, the majority (89.2%) of eggs belonged to *A. fallax*. Two samples (1.1%) were *A. alosa* and 9.7% were hybrids. However, species identification using this method is only considered to be 90% accurate (Faria *et al*. 2011).

- The six microsatellite loci developed by Faria *et al*. (2004) indicated that the Tywi population is genetically different from the Usk and the Wye. However, this result was only found in two of the three analyses. It is considered that the development of additional microsatellite markers should assist in resolving this ambiguity.

Using mitochondrial and nuclear markers, most hybrids were observed to exhibit an *A. fallax* nuclear marker and an *A. alosa* mitochondrial marker, thus indicating hybridization between *A. fallax* males and *A. alosa* females. The number of individual hybrids differed between rivers from 72% in the Tywi to 46% and 29% in the Usk and the Wye respectively. This result could be due to recent or past hybridization, but

based on the observed frequency of the adults in recent decades, it is considered likely that the presence of *A. alosa* in the current samples represents a legacy. In order to enhance the temporal understanding of genetic introgression additional genetic markers would be required. Using the CytB and the nif1-nDNA markers, one *A. alosa* egg was found in the River Wye. This result is very interesting as there are no known spawning sites for this species in Britain even though adult *A. alosa* are regularly found in the UK (Maitland & Hatton-Ellis, 2003). It is recommended that further work on the Lower Wye sampling site (X1) should be carried out to confirm this result. However, CytB and nif1-nDNA are not absolutely reliable species markers and hybridization frequency cannot be assessed with only two markers as hybridization can take place in the entire genome.

Another objective of this project was to genetically identify non-shad eggs. The use of universal primers gave excellent results. These could be routinely applied for species identification. In the field, 15 % of eggs were misidentified as shad. The use of universal primers confirmed that these eggs belonged to minnow *Phoxinus phoxinus* and chub *Squalius cephalus*.

9.2. Future Work

Quality Assurance of Kick Sampling

This project has contributed to the existing shad monitoring programme by confirming that the majority of eggs collected were correctly identified as belonging to shad. However, a small but nonetheless significant number of eggs were misidentified by field surveyors. Investigation of whether improved field protocols can be developed is recommended in order to reduce these errors, for example using taxonomic guidance and further training of field staff. The continued use of barcoding should also be applied routinely to validate correct identification and monitor the accuracy of egg identification in the field. These recommendations are particularly important for verification of potential new shad spawning sites.

Species Identification

For Welsh shad, no single genetic marker exists that can reliably distinguish between *A. alosa, A. fallax* and hybrids, and there is strong evidence of past and/or present hybridisation in all populations. Accordingly, the development of absolute species markers is recommended. The use of next generation sequencing technology will provide the fastest and most cost effective methods to generate a reference dataset of species markers. Once these data are gathered, a Single Nucleotide Polymorphism (SNP) array should be developed. This will provide a rapid assessment tool to infer hybridization routinely in the lab. The development of such a dataset will be essential to determine the level of hybridization in Welsh shad populations. Use of SNPs will also allow the monitoring of shad populations at a fine scale, and infer genetic variability and population sustainability. In turn, this approach could provide the evidence base needed to better support species and conservation management decisions.

10. Acknowledgements

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12. Appendices

12.1. Appendix A: Sampling procedure for Shad genetic work

1- Health and Safety[1]:

Laboratory gloves should be worn during egg sampling to avoid DNA contamination and possible irritation due to chemical (ATL buffer and 70% Ethanol). ATL buffer will digest biological tissue. Do NOT allow any ATL buffer or ethanol to reach ground water, water course or sewage system. There is a danger to drinking water even if small quantities leak into the ground. The risk of contact with eyes is minimal, appropriate eye safety gear should be worn at all times. Eyewash or tap water should be easily accessible.

In the case of:

- Skin contact: Remove/Take off all contaminated clothing immediately. Rinse skin with water/shower.
- Eyes contact: rinse immediately with plenty of water using eyewash or tap water and seek medical advice immediately.
- Ingestion: If swallowed, do not induce vomiting: seek medical advice immediately and show the chemical label.

For further information, please read carefully the health and safety data sheet for these 2 chemicals.

2- Sampling procedure:

In addition to normal sampling equipment, you will require the following:

- Pre-labelled 1.5ml Eppendorf tubes containing ATL buffer (25 per site - supplied);
- 15ml Falcon tubes containing 70% ethanol (supplied);
- Petri dish (supplied);
- Distilled water (supplied);
- Clean forceps;
- Coolbag and coolpack, or a polystyrene box with ice;
- Disposable laboratory gloves;
- Permanent marker (fine).

The Eppendorf tubes will be labelled as follows: A1 – 01

A1: being the sampling site number where the first letter denotes the river (W for Wye, U for Usk, T for Tywi) and the number denotes the sampling site[2]. Survey sites should be numbered in downstream order. Individual survey leaders are responsible for allocating numbers to survey sites and ensuring these are consistently used.

[1] In addition to the Health and Safety procedures listed here, NRW staff were required to follow standard working near water fieldwork procedures.

[2] Due to lack of space on Eppendorf tubes, existing site codes cannot be used.

Sampling date should not be added to the eppendorf tubes but should be written on the recording form. If a label comes off any tubes, these should be re-labelled using the permanent marker on the lid and the side of the tube.

01: being a two digit individual egg number

Falcon tubes will be labelled only with the sampling site number.
You should carry out shad egg kick sampling following the normal procedure; however, instead of returning sampled eggs to the river, they should be collected following the procedure below. When collecting, you should so far as possible record those eggs that you would have counted during normal shad sampling. Doubtful eggs may be sent if you record them as shad eggs on the recording form, but if you are confident that any material collected is not a shad egg, do not send it as this will artificially inflate the number of misidentified eggs. Likewise, do not exclude material that you would in the past have included as a shad egg.

Gloves must be worn at all times to prevent contamination and for safety reasons.

1. Place all eggs in a Petri dish (provided) and rinse with distilled water (provided).
2. Wipe the forceps clean with ethanol. Carefully place one egg into the first Eppendorf tube and close the tube, taking care to avoid contamination by foreign material. Wipe the forceps again with ethanol. Repeat until up to 25 eggs have been collected or until no more eggs are found according to the normal sampling protocol rules. The ATL buffer will start to digest these eggs, so it is very important that only one egg is stored per Eppendorf tube.
3. Place Eppendorf tubes on ice or in coolbags with coolpack.
4. If significantly more than 25 eggs are encountered, additional material (**up to 25 extra eggs**) should be collected and stored together in the Falcon tube containing 70% ethanol. (This will not destroy the cell, so multiple eggs can be stored together, however DNA can be more difficult to extract from eggs stored in this way).
5. On the recording form, mark the numbers of the Eppendorf tubes used (e.g. U1-01 to U1-18).
6. On return from fieldwork, send eggs by next day courier to the address below, together with a copy of the completed recording form (you should retain a copy for our records). The package must be labelled as perishable. If it is not possible to despatch the eggs immediately, they should **be stored in the fridge overnight at 4˚C**.

12.2. Appendix B: DNA quantification:

Population	Sample	Quantity (ng/µl)	260/280
T3	T3-01	1	1.95
	T3-02	86.1	2.06
	T3-03	81.9	2.05
	T3-04	89.3	2.07
	T3-05	123.5	2.09
	T3-06	118.6	2.09
	T3-07	135.8	2.09
	T3-08	59.4	2.03
	T3-09	116.5	2.07
	T3-10	76.6	1.86
	T3-11	62.6	2.01
	T3-12	61	2.04
	T3-13	94.4	2.08
	T3-14	1.8	1.25
	T3-15	40.8	2.06
	T3-negative	0	0
Population	Sample	Quantity (ng/µl)	260/280
W2	W2-01	122.6	1.7
	W2-02	43.5	2.04
	W2-03	43.4	1.98
	W2-04	45.7	1.97
	W2-05	37.6	2.05
	W2-06	37.5	2.01
	W2-07	38.2	1.99
	W2-08	33.5	2.05
	W2-09	41.4	2.02
	W2-10	35.7	2.01
	W2-11	42.2	1.95
	W2-12	39.6	2
	W2-13	43.6	1.98
	W2-14	42.7	1.97
	W2-15	32.6	2
	W2-negative	0.2	0.34
Population	Sample	Quantity (ng/µl)	260/280
W1	W1-01	36.5	2.02
	W1-02	59.1	1.72
	W1-03	14	1.39
	W1-04	95.4	1.64
	W1-05	247	1.62
	W1-06	69.1	1.77
	W1-07	44.1	1.83

	W1-08	62.4	1.58
	W1-09	35.2	2.1
	W1-10	135.2	1.58
	W1-11	37.3	1.67
	W1-12	71.2	1.77
	W1-13	42.5	1.69
	W1-14	52.5	1.85
	W1-15	80	1.64
	W1-negative	0.1	0.2

Population	Sample	Quantity (ng/µl)	260/280
W3	W3-01	28	2.04
	W3-02	145.1	1.6
	W3-03	176.8	1.62
	W3-04	65.4	1.72
	W3-05	65.4	1.92
	W3-06	67.2	1.66
	W3-07	50.7	1.67
	W3-08	66.7	1.81
	W3-09	80.2	1.68
	W3-10	50.2	1.78
	W3-11	131.7	1.66
	W3-12	62	1.68
	W3-13	86.7	1.75
	W3-14	104.5	1.6
	W3-15	41.7	1.78
	W3-negative	0	0.1

Population	Sample	Quantity (ng/µl)	260/280
T1	T1-01	26.3	1.73
	T1-02	139.9	1.56
	T1-03	90.8	1.6
	T1-04	104.8	1.69
	T1-05	195.9	1.53
	T1-06	97.2	1.66
	T1-07	89.5	1.63
	T1-08	106.4	1.66
	T1-09	129.7	1.57
	T1-10	179.6	1.86
	T1-11	153.5	1.55
	T1-12	50.2	1.62
	T1-13	79.9	1.65
	T1-14	32	1.76
	T1-15	155.7	1.54
	T1-negative	0	0.1

Population	Sample	Quantity (ng/µl)	260/280
T2	T2-01	56.7	1.75
	T2-02	177.7	1.73
	T2-03	86.5	1.88
	T2-04	107.2	1.56
	T2-05	115.2	1.61
	T2-06	78.1	1.83
	T2-07	145.4	1.67
	T2-08	93.5	1.7
	T2-09	26.2	1.44
	T2-10	19.6	1.44
	T2-11	92.5	1.92
	T2-12	27.1	1.67
	T2-13	75	1.49
	T2-14	47.5	1.94
	T2-15	120	1.66
	T2-negative	0	0.2

Population	Sample	Quantity (ng/µl)	260/280
U2	U2-01	29.3	2.02
	U2-02	36.6	2.02
	U2-03	1.7	1.59
	U2-04	40.2	2.04
	U2-05	26.2	2.08
	U2-06	6.8	2.16
	U2-07	14.3	1.79
	U2-08	21.1	1.92
	U2-09	24.6	2.06
	U2-10	16.3	2.01
	U2-11	27.8	2.03
	U2-12	6.1	2.03
	U2-13	32	2.1
	U2-14	16.9	2.18
	U2-15	11.2	1.92
	U2-negative	0	0.1

Population	Sample	Quantity (ng/µl)	260/280
U3	U3-01	60.9	2.02
	U3-02	7.5	1.99
	U3-03	55.1	1.66
	U3-04	44.6	1.95
	U3-05	7.3	1.93
	U3-06	25.1	1.92

	U3-07	55.1	2.07
	U3-08	1.2	1.54
	U3-09	53.1	2.01
	U3-10	33	1.99
	U3-11	31.7	1.97
	U3-12	42.6	1.98
	U3-13	32.4	1.96
	U3-14	51.7	2.02
	U3-15	27.8	1.94
	U3-negative	0	0.1

Population	Sample	Quantity (ng/µl)	260/280
U4	U4-01	31.6	2.05
	U4-02	28.8	2.01
	U4-03	77.8	1.78
	U4-04	16.3	1.38
	U4-05	70.1	2.02
	U4-06	77.9	1.76
	U4-07	61.2	1.79
	U4-08	48	1.93
	U4-09	34.8	1.76
	U4-10	27.7	1.74
	U4-11	19.1	1.73
	U4-12	16.6	1.78
	U4-13	47.4	1.9
	U4-14	22.8	1.82
	U4-15	14.8	1.51
	U4-negative	0.4	1.15

Population	Sample	Quantity (ng/µl)	260/280
W4	W4-01	6.8	1.37
	W4-02	30.4	1.86
	W4-03	34.9	1.54
	W4-04	37	1.86
	W4-05	34.9	1.7
	W4-06	36.9	1.82
	W4-07	29.8	1.71
	W4-08	27.5	1.68
	W4-09	23.5	1.87
	W4-10	29.3	1.87
	W4-11	31.3	1.8
	W4-12	45.2	1.8
	W4-13	21.6	1.88
	W4-14	54.9	1.8
	W4-15	49.4	1.89

	W4-negative	0	0.1

Population	Sample	Quantity (ng/µl)	260/280
X1	X1-01	42.9	1.73
	X1-02	117.6	1.99
	X1-03	57.2	2
	X1-04	75	2.02
	X1-05	72.5	2.09
	X1-06	12.3	1.4
	X1-07	73	2.02
	X1-08	19.7	1.79
	X1-09	29.7	1.82
	X1-10	102.7	1.95
	X1-11	79.6	2.12
	X1-12	63.2	1.92
	X1-13	40.9	1.91
	X1-14	67.8	1.89
	X1-15	116.3	1.91
	X1-negative	0	0.2

12.3. Data Archive Appendix

Data outputs associated with this project are archived as project 436, media 1473 on server–based storage at Natural Resources Wales.

The data archive contains:

[A] The final report in Microsoft Word and Adobe PDF formats.

[B] Electronic copies of the field sheets, saved as pdfs.

[C] An Excel spreadsheet Ninf_mito_microsat_scoring (NRW-13-066287) detailing the RFLP, mtDNA and microsatellite results for each egg.

[D] Three FASTA format files, TreeUniveralPrimers2.fas (NRW-13-084552); Ninf_sequences.fas (NRW-13-066284) and Shad_CytochromeB_all2.fas (NRW-13-066283).

Metadata for this project is publicly accessible through Natural Resources Wales' Library Catalogue http://194.83.155.90/olibcgi by searching 'Dataset Titles'. The metadata is held as record no 115546.

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