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SHORT COMMUNICATION

Genetic data confirm the presence of juvenile *Alosa alosa* in the estuary of the River Tamar

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Abstract

Within the UK, allis shad (*Alosa alosa*) are classified as Critically Endangered and are known to breed in only a single river, the Tamar. Despite evidence of spawning within the lower freshwater reaches of the river and at the tidal limit within the estuary, juvenile allis shad have never been fond. Genetic analysis, based on mitochondrial DNA haplotype and nuclear Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) data, confirmed that juvenile shad found within the lower Tamar estuary in autumn 2022 were *A. alosa*.

KEYWORDS
allis shad, anadromy, hybrid, mitochondrial DNA

The shads *Alosa alosa* (allis shad) and *A. fallax* (twaite shad) are two anadromous clupeid species, with widespread distributions along the western European Atlantic and Mediterranean coasts. Within the UK, both species have declined in recent years and populations have been extirpated from many rivers where they were once found (Hillman, 2003; Maitland & Hatton-Ellis, 2003). Historically more widespread, allis shad (*A. alosa*) are known to breed in only a single UK river, the Tamar (Hillman, 2020). Despite having been given considerable legal protection under European Union directives and UK Acts of Parliament (Maitland & Hatton-Ellis, 2003), numbers of individuals have continued to decline and the species is considered Critically Endangered and threatened with extinction in Britain (Nunn et al., 2023). Adult allis shad are occasionally trapped in a fish trap on Gunnislake Weir at the tidal limit of the River Tamar, as they attempt to migrate upstream. Spawning is known to take place at three sites approximately 2 km upstream of Gunnislake Weir and at two sites within a 0.3 km extent downstream of the tidal limit (Cotterell & Hillman, 2016; Hillman, 2020). However, juvenile shad have never been found.

In October 2022, Marine Natural Capital and Ecosystem Assessment (mNCEA) netting surveys found juvenile clupeids at two sites within the Tamar estuary. These sites are approximately 12 km (Higher Braunder) and 16.5 km (Weir Quay) downstream of known allis shad spawning sites. Based on morphology and gill raker counts, these juveniles appeared to be allis shad. It is thought that this is the first report of juvenile allis shad having been found utilizing the upper/middle Tamar estuary as a nursery habitat.

We undertook genetic analysis, based on sequencing of maternally inherited mitochondrial DNA (mtDNA) and restriction analysis of a biparentally inherited nuclear DNA fragment to elucidate the species identity of the three juvenile shad and place the results in a wider context by comparing the data to adult shad caught in the Tamar and a marine area adjacent to the Tamar estuary.

The work represented here did not require ethics approval. Tissue samples of *Alosa* sp. were available from three different sources (Table 1). Specimens of three juvenile shad, tentatively identified as allis shad on the basis of gill raker counts (Aprahamian et al., 1998), were sampled during seine netting surveys within the Tamar estuary.

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TABLE 1 Details of *Alosa* sp. samples.

Location	Code	Species	Gill raker counts	Grid ref[b]	Sampling date	Length (mm)	Weight (g)	ND1 haplotype	*nif-nDNA/Hae*III result
Higher Braunder	FRY37	*A. alosa*	nd	50.484, −4.218	6th Oct 2022	37	–	Aa5	*A. alosa*
	FRY43	*A. alosa*[a]	36	50.484, −4.218	6th Oct 2022	43	–	Aa5	*A. alosa*
Weir Quay	FRY60	*A. alosa*[a]	42	50.467, −4.215	7th Oct 2022	60	–	Aa5	*A. alosa*
Gunnislake Weir	40630	*A. alosa*	nd	50.519, −4.206	23rd Jun 2021	490	–	Aa1	*A. alosa*
	41009	*A. alosa*	nd	50.519, −4.206	7th Jul 2021	421	–	Aa5	*A. alosa*
	41616	*A. alosa*	nd	50.519, −4.206	17th May 2022	409	–	Aa5	*A. alosa*
	41617	*A. alosa*	nd	50.519, −4.206	17th May 2022	436	–	Aa5	*A. alosa*
	41686	*A. alosa*	nd	50.519, −4.206	6th Jun 2022	504	–	Aa5	*A. alosa*
	41703	*A. alosa*	nd	50.519, −4.206	7th Jun 2022	459	–	Aa5	hybrid
	41789	*A. alosa*	nd	50.519, −4.206	13th Jun 2022	420	–	Aa5	*A. alosa*
	41802	*A. alosa*	nd	50.519, −4.206	13th Jun 2022	405	–	Aa5	*A. alosa*
	42112	*A. alosa*	nd	50.519, −4.206	30th Jun 2022	479	–	Aa5	*A. alosa*
	310522	*A. alosa*	nd	50.519, −4.206	31st May 2022	471	–	Aa5	*A. alosa*
	050722_1	*A. alosa*	nd	50.519, −4.206	5th Jul 2022	500	–	Aa5	*A. alosa*
	050722_2	*A. alosa*	nd	50.519, −4.206	5th Jul 2022	473	–	Aa5	*A. alosa*
	060722_1	*A. alosa*	nd	50.519, −4.206	6th Jul 2022	455	–	Aa5	*A. alosa*
	060722_2	*A. alosa*	nd	50.519, −4.206	6th Jul 2022	495	–	Aa5	*A. alosa*
	070722_1	*A. alosa*	nd	50.519, −4.206	7th Jul 2022	524	–	Aa5	*A. alosa*
	070722_2	*A. alosa*	nd	50.519, −4.206	7th Jul 2022	470	–	Aa5	*A. alosa*
Whitsand Bay	C-SH-1	*A. fallax*[a]	43	50.317, −4.228	6th May 2021	414	1040	Af3	*A. fallax*
	C-SH-2	*A. alosa*[a]	82	50.327, −4.234	11th Jun 2021	456	1405	Aa5	hybrid
	C-SH-3	*A. fallax*[a]	40	50.319, −4.231	3rd Nov 2021	382	960	Af21	*A. fallax*
	C-SH-4	*A. fallax*[a]	38	50.319, −4.231	3rd Nov 2021	405	945	Af21	*A. fallax*
	C-SH-5	*A. fallax*[a]	39	50.323, −4.235	13th Nov 2021	370	910	Af21	*A. fallax*

Note: Mitochondrial NADH dehydrogenase subunit I (ND1) gene haplotypes and results for *nif-nDNA/Hae*III digest are also given.
Abbreviation: nd, not determined.
[a]Species determination based on gill raker counts.
[b]Grid references are latitude and longitude given in decimal degrees.

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Reference adult samples were scale samples taken from 16 allis shad caught in a fish trap associated with a fish pass located on a weir at the tidal limit of the Tamar at Gunnislake, Cornwall, and five specimens (one *A. alosa* and four *A. fallax*) from Whitsand Bay, caught as by-catch during marine netting activities for the SAMARCH sea trout project (www.samarch.org). These five specimens were assigned to species based on gill raker counts (Tea Bašić, CEFAS, personal communication 2023).

DNA was extracted using Qiagen DNeasy Blood and Tissue kits. An approximately 1250 bp region of mtDNA that includes the NADH dehydrogenase subunit I (ND1) gene was amplified as described in Faria et al. (2006). To determine the hybrid nature of each sample, we amplified an approx. 380 bp fragment of nuclear DNA (*nif1-nDNA*) as described in Faria et al. (2011). The two species are distinguished by the presence/absence of a *Hae*III restriction site (Hardouin et al., 2013). PCR products were digested with *Hae*III following Hardouin et al. (2013) and visualized on ethidium bromide-stained 1.5% agarose gels.

ND1 PCR products were purified using Qiagen PCR Purification kits and sequenced via Sanger sequencing in both forward and reverse directions by Eurofins. Sequences were edited in BioEdit v7.2 (Hall, 1999) and aligned in Clustal X v1.83 (Thompson et al., 1997). Basic analyses (number of haplotypes and number of individuals per haplotype) and haplotype network construction were undertaken using the R packages pegas v1.2 (Paradis, 2010) and ape v5.7.1 (Paradis & Schliep, 2019).

To set the haplotypes found in Tamar shad in a wider European context, previously published *A. alosa* and *A. fallax* ND1 sequences (Faria et al., 2012) were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov), restricted to haplotypes found in Atlantic populations of the two species, resulting in a total of 10 and 20 haplotypes for *A. alosa* and *A. fallax*, respectively. A neighbour-joining dendrogram, based on Kimura 2-parameter (Kimura, 1980) distance between haplotypes, was constructed using MEGA v6 (Tamura et al., 2013).

The full sequence of the mtDNA ND1 gene (975 base pairs) was obtained from all 24 samples. In total, 36 base pairs were polymorphic, identifying four distinct haplotypes (GenBank accession numbers OR829725-OR829748, Figure 1). Basic Local Alignment Search Tool (BLAST) analysis showed that all four haplotypes are identical to haplotypes previously identified in *A. alosa* and *A. fallax* (Faria et al., 2012).

The three Tamar Estuary juveniles possessed the Aa5 *A. alosa* haplotype, also found in the majority of the adult shad sampled at Gunnislake (Figure 1a). This *A. alosa* Clade 1 haplotype (Figure 1b) was the most commonly found by Faria et al. (2012), being present in Portuguese, French and Scottish samples of allis shad. The Aa1 haplotype was found in a single individual sampled at Gunnislake weir (Table 1). Interestingly, this *A. alosa* Clade 2 (Figure 1b) haplotype was found by Faria et al. (2012) in only a single *A. alosa* individual sampled from the Dordogne River in south west France. The other haplotype in this clade (Aa2) was found in the Solway Firth and western French allis shad populations. Using nuclear microsatellite markers, allis shad populations in western France and the UK belong to the same genetic group (Sabatino et al., 2022), suggesting that there may be the possibility of extensive gene flow between *A. alosa* populations in this part of their range.

Two ND1 haplotypes were found in the four *A. fallax* individuals, with three possessing Af21 and one Af3. Haplotype Af21, belonging to *A. fallax* Clade 1 (Figure 1b), is the most common haplotype in Atlantic populations of *A. fallax* (Faria et al., 2012), while haplotype Af3 (Clade 2) was also found in the North Sea and Bristol Channel (Severn & Wye) *A. fallax* populations.

Restriction digestion of the *nif1-nDNA* fragment identified the three juveniles as allis shad (*A. alosa*) (Table 1 and Figure 1c). The majority of adults sampled at Gunnislake were also allis shad, with a single individual identified as a potential hybrid. Of the five fish sampled in Whitsand Bay, four were pure twaite shad with the individual identified as an allis shad based on gill raker counts, being of hybrid origins (Table 1).

There was no evidence of introgression of *A. fallax* mtDNA into any of the Tamar *A. alosa* specimens studied here. Hybrids between the two shad species are common with extensive sharing of mtDNA haplotypes being reported (Faria et al., 2012; Taillebois et al., 2020), including for River Tamar allis shad (Jolly et al., 2012). Hybrid individuals, based on nuclear microsatellite genotypes, have been identified in the Tamar (Antognazza et al., 2022; Jolly et al., 2012). Taillebois et al. (2020) found that the general direction of introgression of mtDNA haplotypes and nuclear DNA alleles was from *A. fallax* towards *A. alosa*. The ability to detect hybrids on the basis of a single nuclear marker (e.g. *nif1-nDNA*) will be limited and further screening with a more extensive nuclear data set (i.e. Taillebois et al., 2020) will be necessary to fully uncover the extant of hybridization in the current River Tamar allis shad population.

Multiple factors contribute to the allis shad population in the Tamar being threatened with extinction. The potentially very small adult spawning run size, multiple, significant migratory barriers and the discrete location of spawning aggregations make the population particularly susceptible to extinction (Hillman, 2020; Nunn et al., 2023). In addition, given the evidence of hybridization between the two shad species in the Tamar (Antognazza et al., 2022; Jolly et al., 2012), genetic swamping (e.g. Roberts et al., 2010), via hybridization with *A. fallax*, which is more abundant in UK waters, is also a potential threat to the genetic integrity of Tamar allis shad.

Despite these issues, this work represents the first report of successful spawning and development into juvenile fish within the Tamar estuary, suggesting that not only are allis shad spawning in the Tamar, but that resulting juveniles are able to utilize the estuary to complete their lifecycle. These results will help to inform management decisions within the estuary as they reveal for the first time the location and seasonality of allis shad nursery areas within the estuary.

Understanding that not only are juvenile *A. alosa* present and using the Tamar estuary, but when they are doing so is extremely valuable information. This evidence can help inform management decisions of existing and future activities within the estuary, particularly those

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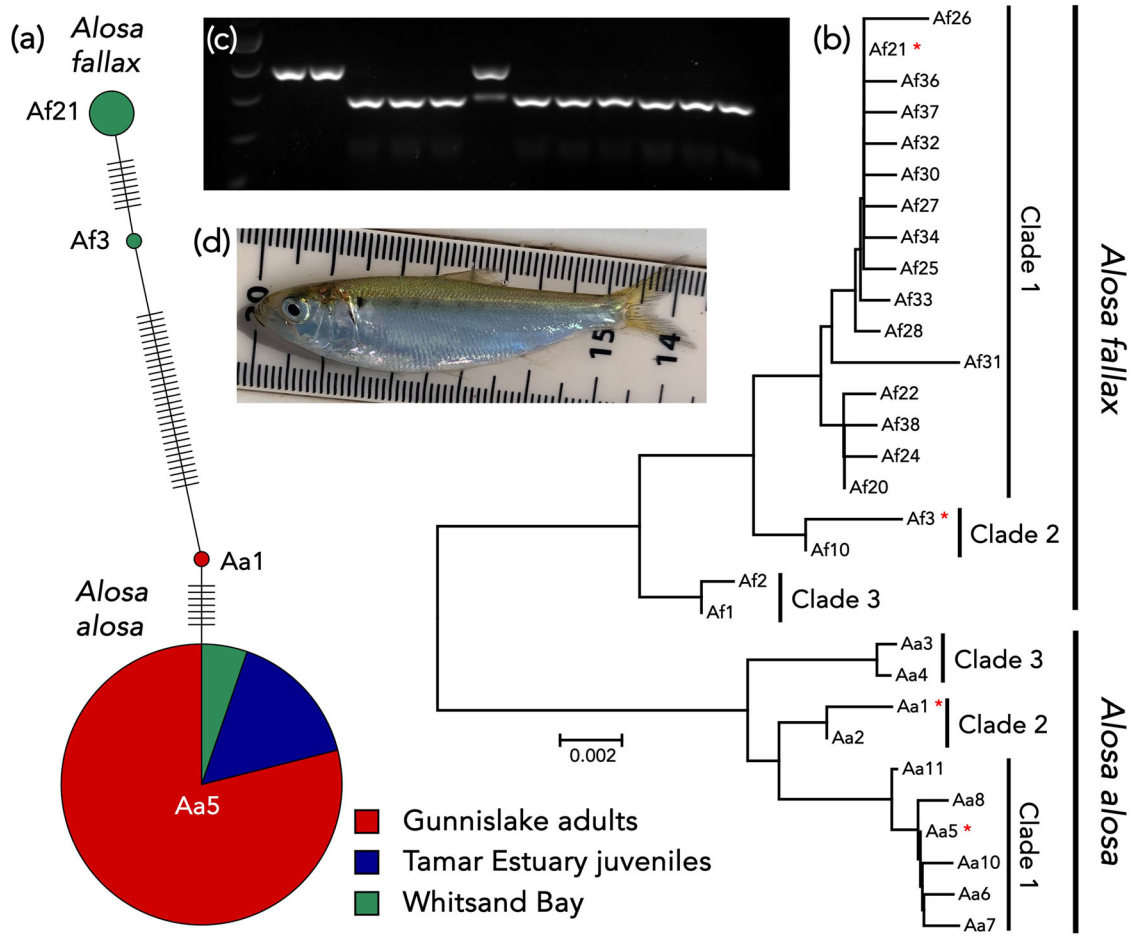

FIGURE 1 (a) Haplotype network for the four mitochondrial NADH dehydrogenase subunit I (ND1) haplotypes found in Tamar *Alosa* sp. The size of symbol is proportional to haplotype frequency and is colour-coded by sample location. Branch lengths are proportional to the number of nucleotide differences between haplotypes. Each dash on connecting lines represents one nucleotide difference. Haplotype names follow Faria et al. (2012). (b) Neighbour-joining dendrogram, based on Kimura 2-parameter (Kimura, 1980) distances between ND1 haplotypes, found in Atlantic populations of *Alosa alosa* and *A. fallax* by Faria et al. (2012). Haplotypes found in River Tamar and Whitsand Bay samples are indicated with a red asterisk. Clade names follow Faria et al. (2012) (c) *nif-nDNA/Hae*III digest results for 12 shad samples. Lane 1: 100 bp ladder (New England Biolabs), Lanes 2 and 3: Whitsand Bay *A. fallax*, Lanes 4–6: Tamar estuary shad fry, Lane 7: Gunnislake adult hybrid, Lanes 8–12: Gunnislake adult allis shad. (d) Photograph of Tamar estuary juvenile shad (FRY60).

occurring around sensitive periods in the shad lifecycle that could have an adverse effect on the Tamar population.

AUTHOR CONTRIBUTIONS

R. Andrew King: Data curation; formal analysis; investigation; writing—original draft. **Rob Hillman**: Investigation; resources; writing—review and editing. **Jay Boyle**: Conceptualization; funding acquisition; writing—review and editing. **Jamie R. Stevens**: Conceptualization; funding acquisition; project administration; resources; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank/NCBI at https://www.ncbi.nlm.nih.gov under accession numbers OR829725-OR829748.

PEER REVIEW

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