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Hybridisation and genetic population structure of *Alosa* population in United Kingdom

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Abstract

Human-mediated habitat fragmentation has been proposed as the main factor driving hybridization between the sympatric migratory European shads *Alosa alosa* and *Alosa fallax*, which has co-occurred with substantial population declines in *A. alosa*. In river systems across Great Britain, shad are negatively affected by navigation weirs constructed in the last 150 years that impede their spawning migrations. Consequently, the aim here was to assess the impact of human disturbances on genetic introgression and population structure of shad in Great Britain through genotyping 119 *Alosa* spp. using 24 microsatellite loci.

Keywords: *Alosa* spp., hybrids, introgression, microsatellite.

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Conflict of interest

The authors declare no conflict of interest.

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Brief communication

The European shads *Alosa alosa* (L., 1758) and *Alosa fallax* (Lacepède, 1803) are anadromous fishes that have undergone substantial population declines in recent years and so are now afforded conservation protections at national and international levels (e.g. Jolly *et al*.,2012). These anadromous clupeids have broad, largely overlapping distributions across the north-east Atlantic (Baglinière *et al*., 2003; Aprahamian *et al*., 2003), that has resulted in the two species extensively hybridizing in European rivers during the last 150 years. Their hybridization has been aided by their shallow inter-specific genetic divergence and sharing of similar life histories, plus their hybrids being reproductively viable (Quignard & Douchement, 1991; Jolly *et al*., 2011). This has resulted in high levels of genetic introgression between the two species, although the extent of hybridisation varies considerably across different rivers in their range (e.g. Aprahamian *et al*.,2003; Alexandrino *et al*., 2006; Coscia *et al.,* 2010; Jolly *et al*., 2011; Faria *et al*., 2012).

The reported high rates of genetic introgression have resulted in shad with intermediate morphological traits and mitochondrial DNA haplotypes from both species (Baglenière *et al*., 2003; Faria *et al*., 2012). In Great Britain, evidence suggests that spawning *A. alosa* are now restricted only to the Solway Firth (on the boundary between England and Scotland) and River Tamar (south-west England) (Jolly *et al*., 2012), while *A. fallax* populations persist only in rivers draining into the Bristol Channel, having disappeared from rivers such as the River Thames (Aprahamian & Aprahamian, 1990; Maitland & Lyle, 2005). Knowledge on the extent of hybridisation rates between the two European shad species have, however, been limited by the extent of genetic markers available that can distinguish between the two species and their hybrids. For example, the most recent studies completed using British shad populations relied on mitochondrial DNA and nuclear DNA using only five microsatellite

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loci (Jolly *et al*., 2012). Consequently, to derive enhanced understandings of the extent of genetic introgression of European shad populations in British rivers, and their extent of their population structuring, 24 microsatellite loci are used here. The results from these analyses should thus provide increased insights into the contemporary status of these fishes and so provide more reliable recommendations to conservation policies and practices.

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) from scales and eggs following the manufacturer's instructions. Scales were available from mature adults sampled from the Solway Firth, River Tamar (n= 44) and the River Severn (Table 1; Figure 1a), with scales also available from some over-wintering shad captured by fishermen as by catch in Poole Bay on the south coast of England (Table 1; Figure 1a). In addition, the deployment of drift nets downstream of spawning grounds provided shad eggs for analysis from the River Tywi, River Tamar (n = 16) and the River Severn (n = 1) (Table 1). In order to determine the level of hybridisation between *A. alosa* and *A. fallax* in Great Britain, these populations (total number of individuals n = 119) were combined with a collection of tissues from individuals of three different populations from the Minho River (Portugal), that previously had been both morphologically and genetically characterized with 24 microsatellite loci (Sabatino *et al.,* 2021). Specifically, one population was identified by *A. alosa* individuals, one by *A. fallax* individuals and the third one by hybrid individuals (Table 1). The inclusion of the pure and hybrid populations was critical in allowing the identification of hybrids in British populations, for which morphological information was lacking. The previously designed microsatellite loci (n = 24) for *A. alosa*, *A. fallax* (Faria *et al*., 2004) or *Alosa sapidissima* (Waters *et al*., 2000; Julian & Bartron, 2007), used for genotyping Minho populations were used to genotype each sample as per (Sabatino *et al.,* 2021). Polymerase chain reaction (PCR)

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amplifications and following steps were according to (Sabatino *et al.,* 2021) (Supplementary information S1).

Genotyping artefacts were assessed using Microchecker version 2.2.3 (Van Oosterhout *et al*., 2004). Departure from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and inbreeding coefficients (F_{IS}) (Weir and Cockerham 1984) were assessed for each locus-population combination and for each locus across all populations using Genepop 4.0.10 (Rousset, 2007) with 10,000 permutations of the data.

Admixture analysis was conducted to assess the hybrid status of UK individuals, using the Bayesian clustering methods implemented in STRUCTURE version 2.3.4 (Pritchard *et al*., 2000; Falush *et al*., 2003) and NewHybrids version 1.0 (Anderson & Thompson, 2002), as detailed in Supplementary Information S2. The cut off to determine hybrids individuals was set at $q \geq 0.99$. Identified hybrids were then removed from the dataset to perform population genetic structure analysis. The pure individuals were selected by removing any individuals with less than 99% chance of being pure. This resulted in the following number of pure *A. fallax*: Poole Bay (9/9), Severn (12/22), Solway Firth (4/15), Tywi (4/13), Minho_ SV (30/41), Minho1 (3/41). The number of pure *A. alosa* identified was Tamar (25/60), Solway Firth (3/15), Minho_MNR (12/14). Due to the restricted sample size of some populations after hybrids were removed, further population genetic analyses were only carried out only on Tamar and Minho_MNR populations, and on Poole Bay, Severn, and Minho_SV, for *A. alosa* and *A. fallax*, respectively. ~~The resulting datasets were then screened as per initial dataset and only these results are reported.~~ Departure from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and inbreeding coefficients (F_{IS}) were assessed as described above for the resulting datasets without any potential hybrid. Genetic structuring between the populations was then explored by calculating pairwise F_{ST} (Weir and Cockerham 1984) using FSTAT 2.9.3.2 (Goudet 1995) with 10,000 permutations. To visualise the relationships

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among individuals, based on their multi-locus allele frequencies, factorial correspondence analysis (FCA) was performed using GENETIX 4.05 (Belkhir *et al*., 2004). Genetic diversity, estimating the number of alleles (A), and observed and expected heterozygosity (H_O and H_E, respectively), using FSTAT 2.9.3.2 (Goudet, 1995). Genetic substructure and admixture were further explored using STRUCTURE 2.2 (Pritchard *et al*., 2000), varying the number of clusters (K) between one and number of populations analysed +2, using the admixture model with default parameters and without using location as a prior. Ten replicates of STRUCTURE were run per K using a burn-in of 150,000 iterations followed by 750,000 additional iterations. Each K was evaluated using the likelihood (Pritchard *et al*., 2000) and ΔK (Evanno *et al*., 2005) methods and summarised and visualised using CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) and DISTRUCT version 1.1 (Rosenberg, 2004), respectively.

The results revealed a high level of hybridisation (52% overall) between *A. alosa* and *A. fallax* throughout Great Britain, with hybrids being detected in all sampled areas except the over-wintering fish sampled from Poole Bay, where all fish were classed as *A. fallax*.

The majority of *A. alosa* individuals were in the River Tamar, and three individuals in Solway Firth (Table 1). Purebred *A. fallax* were not identified in the River Tamar. In the Rivers Severn and Tywi, and Solway Firth, the shad were a mix of hybrids and pure *A. fallax*, where the lowest hybrid proportion was in the River Severn (45.5%) and highest in the River Tywi (69%) (Table 1). The shad hybrids in the River Severn and Tywi mainly clustered towards *A. fallax* (mean proportion of membership of *A. fallax* cluster 0.90 and 0.93, respectively), with a similar trend for Solway Firth but with more variation (mean proportion of membership of *A. fallax* cluster 0.51, and 0.49 *A. alosa* cluster). Hybrids in the River Tamar, however, had a higher membership proportion in the *A. alosa* cluster (mean

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proportion of membership of *A. alosa* cluster 0.97). Complete F1, F2 and backcrosses identification was not possible to perform exhaustively, however, NewHybrids analysis did identify two samples (one from River Severn and one from Solway Firth) as F1 hybrids (concordant with STRUCTURE results) and suggested two samples from Solway Firth as potential F2 hybrids (Supplemental Information S3).

In both *A. alosa* populations, the mean number of alleles per locus was 4.222, with mean heterozygosity between 0.548 and 0.580 (Table 2) and in *A. fallax* populations, the mean number of alleles per locus ranged between 3.178 and 3.706 (Table 2), with mean heterozygosity between 0.389 and 0.504. No linkage disequilibrium was found between the loci. *A. fallax* Poole Bay and Minho_SV populations showed a significant inbreeding coefficient, F_{IS}, being 0.241 and 0.085 respectively (Table 2). Calculations of F_{ST} revealed significant genetic differences between all population comparisons. Specifically, $F_{ST\text{-Poole Bay-Severn}}$ 0.097, $F_{ST\text{-Poole-Bay-Minho\_SV}}$ 0.187, $F_{ST\text{-Severn-Minho\_SV}}$ 0.164, $P < 0.05$, for *A. fallax* populations and, $F_{ST\text{-Tamar-Minho1}}$ 0.061, $P < 0.05$ for *A. alosa* populations. Both Bayesian analysis (Figure 1a-b) and factorial correspondence analysis (FCA) (Figure 1d-e) supported F_{ST} results for both species. Bayesian analysis suggested K=2 as the most probable number of genetic pools for both species (Figure 1b-c). FCA analysis highlighted the genetic differentiation among the two English *A. fallax* populations and the Portuguese one, further indicating a closer relationship between Poole Bay and Severn populations. Between Tamar and Minho1, *A. alosa* populations, a lower genetic differentiation is highlighted and concordant among the analyses (Figure 1b-d).

High level of hybridisation was detected in all sampled areas except the over-wintering fish sampled from Poole Bay, where all fish were classed as *A. fallax*. The increased panel of microsatellite loci adopted in this study (24 vs 5 Jolly *et al.,* 2012), consistently identified

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more hybrids in three of the four populations (53 vs 22% Solway Firth, 58 vs 13% River Tamar, 46 vs 24% River Severn (Jolly *et al.,* 2012). The river Tywi, had comparable hybridisation levels (69 vs. 70%). Where more hybrids were detected, the increase exceeded 50%, with increased levels of hybridisation most probably being the result of the more in-depth screening of the genome when using 24 vs 5 microsatellite loci, as the sample collection of the two studies overlaps. However, we cannot exclude the possibility that hybridisation levels have increased for Severn fish, as samples analysed in this study were collected after seventeen years the samples analysed in Jolly *et al*., (2012) or indeed the possibility that the samples come from related individuals. On the other hand, the F_{IS} level for the rivers with high hybridisation levels do not indicate high levels of inbreeding. Significant F_{IS} levels were only found in Poole Bay fish (pure *A. fallax*) and Minho fish (pure *A. fallax*) (Table 2). The high and significant F_{IS} value for Poole Bay fish could be due to inbreeding or a bottleneck effect, however it might also be due the small sample size of this population, thus further investigations are needed. Minho fish (pure *A. fallax*) displayed a slightly positive significant F_{IS} value which could be due to the small sample size, since data using a higher sample size from the same river and same period do not show significant F_{IS} (Sabatino, *et al.,* 2021).

As per Jolly *et al.,* (2012), the results revealed that *A. alosa* only persist in Britain in the Solway Firth and River Tamar, despite previously being relatively common in inshore areas, especially around the Bristol Channel area, where they used to spawn in rivers such as the Severn (Aprahamian, 1982). Their loss in these inshore areas and spawning rivers has been suggested to be due to the presence of navigation weirs that block their riverine spawning migrations (Maitland & Lyle, 2005). The Bristol Channel area does, however, remain the stronghold for British *A. fallax* populations (Maitland & Lyle, 2005; Noble *et al*., 2007), with the results here revealing that in this area, even the hybrids cluster more strongly with *A.*

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fallax than *A. alosa*. This suggests that in this system, *A. fallax* might always have been the predominant species, with the construction of the anthropogenic structures in the 19th Century then eliminating any remaining pure *A. alosa*.

The presence of anthropogenic structures in the lower reaches of rivers has already been postulated as major factor in the relatively high levels of hybridisation between the two European shad species (e.g. Aprahamian *et al*., 2003). The occurrence of hybrids in high frequencies thus potentially results from these migration blockages forcing both European shad species to utilise the same spawning areas at similar times and losing their spatial segregation (Boisneau *et al.*, 1992; Limburg and Waldman 2009). This is emphasised by the majority of sampled locations in this study being affected by anthropogenic structures with, for example, the Rivers Tamar and Severn having substantial structures in their lower reaches that are known to impede shad movements (e.g. Antognazza *et al*., 2019).

While the River Tywi does not have the same presence of anthropogenic barriers in its lower reaches, 69% of its analysed fish were classed as hybrids. This could be explained by (a) hybridisation between the two shads being a natural phenomenon, irrespective of any river engineering; and as previously suggested by Jolly *et al*., (2012), or (b) sample bias, due to the low number of sampled individuals in most of the populations analysed in this study and/ or (c) since the construction of navigation weirs in the River Severn in the 19th Century, the production of large numbers of viable *Alosa* hybrids has resulted in their spill-over into neighbouring rivers. This latter explanation is at least partially supported by the results of the population structure analyses here that indicated weak genetic structuring in British *A. fallax* populations. Furthermore, the absence of F1 and F2 fish in the Tywi suggests that hybridisation is not recent. There were, however, significant genetic differences between the over-wintering *A. fallax* sampled from Poole Bay and the River Severn populations, suggesting negligible gene flow between these fish. Given the geographic distances between

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these populations, and the relatively close proximity of Poole Bay to European shad spawning rivers in mainland Europe, then these results suggest that *Alosa* spp. that overwinter in inshore areas around Southern Britain are fish that migrate in spring to rivers further south for spawning. Indeed, recent data from telemetry studies on *A. fallax* that were migrating upstream in the River Severn revealed some individuals overwintering in inshore areas around Ireland, with the total distances moved by individual shad between their annual spawning periods being at least 950 km (Davies *et al.,* 2020). However, the low sample size of the Poole Bay sample inhibits further work on this at present. Furthermore, no previous studies on Poole Bay shad populations have been performed, thus more focus on this population is needed to support more in-depth speculations.

Despite the presence of major migratory barriers in the lower reaches of the River Tamar (being in the lowest reach Gunnislake Weir, 5km upstream Duchess Weir and another 5 km upstream Lamerhooe Weir), this river's *A. alosa* population is persisting. Nevertheless, a high level of hybrids (58%) has been detected, highlighting the high conservation importance of this river for *A. alosa* conservation. Pure *A. alosa* were also detected in Solway Firth, although these fish were captured from coastal waters, rather than in the river, with shad spawning locations in the river yet to be identified (Maitland & Lyle, 2005). This is interesting, given that as with many anadromous species, both *A. alosa* and *A. fallax* appear to exhibit some tendency to return to their natal river for spawning (Davies *et al.,* 2020), albeit with some straying (e.g. Waters *et al*., 2000; Jolly *et al*., 2012). However, it is possible that these behaviours are altered by their genetic introgression, resulting in increased straying (Keefer *et al*., 2014).

Given that it is generally accepted that a principal driver in the genetic introgression of European shads is the presence of anthropogenic structures in the lower reaches of their spawning rivers, then their conservation management can focus on efforts to increase river

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connectivity that aims to reduce the probability of hybrid formation. Given the persistence of both *A. alosa* and *A. fallax* in British waters, including *A. alosa* genes in areas where the pure species is currently absent, then increasing river connectivity through modifying these engineered structures should potentially enable the re-establishment of the spatial segregation of the species' spawning areas. For example, on the River Severn, a major restoration project ('*Unlocking the Severn'*) is underway to re-open the entire river to migrating shad through weir modifications, including fish pass installations (Antognazza *et al*., 2019; 2021). Despite the high proportion of *Alosa* spp. hybrids, there are no studies on their fitness, ability to pass anthropogenic structures or their reproductive success. Consequently, it remains uncertain as to whether hybridisation poses a long-term risk to the status of these populations and whether efforts are needed to reduce the detected rates of introgression. By measuring the long-term responses of *Alosa* spp. to the re-opening of the River Severn in their movement behaviours, spawning distributions and genetics (including use of genome wide markers), then deeper insights into the introgressive hybridisation of *Alosa* spp. and in relation to the impacts on this of human disturbances should be generated. Correspondingly, the data developed in this study should provide an important baseline for measuring long-term conservation progress and gains.

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Figure legend

Figure 1: a) Sampling locations across British Isles; b) STRUCTURE bar plot (K=2), highest likelihood run out 10 repetitions for *A. alosa* populations and c) for *A. fallax* populations (K=2, highest likelihood run out 10 repetitions); d) factorial correspondence analysis (FCA) between *A. alosa* population and e) among *A. fallax* populations.

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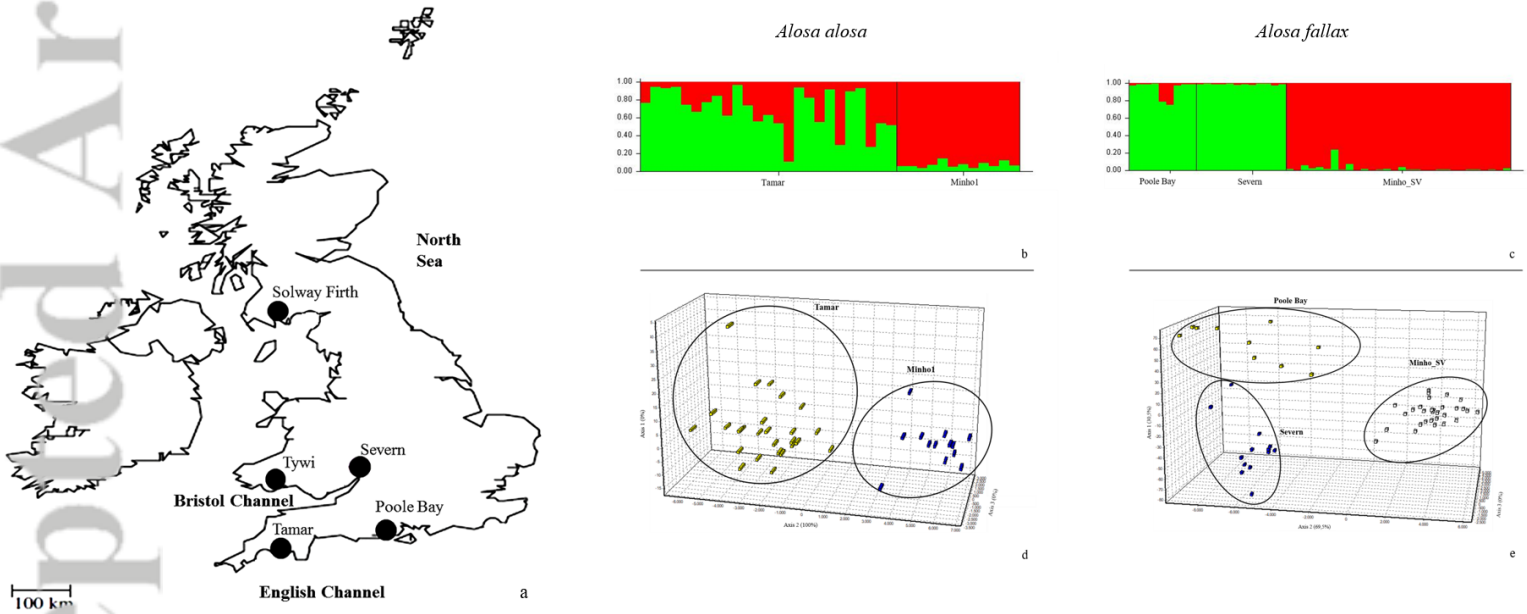

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Significance statement

European anadromous shads have undergone population declines due to habitat fragmentation, leading to hybridisation. In absence of robust morphological data, genetic markers provide a cost-effective method for genetic screening. In British rivers, shad are negatively affected by navigation weirs that impede their spawning migrations. The aim here was to assess the impact of human disturbances on genetic introgression and population structure of shad in Great Britain through genotyping 119 *Alosa* spp. using 24 microsatellite loci.

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Table 1: Description of samples: sampling location, number of individuals (N), country and species assignment based on morphology (Sabatino *et al.,* 2021) are detailed. There was no species assignment for British populations. Individual classification in three groups (*Alosa fallax, A. alosa* and hybrids) based on admixture analyses ($q \geq 0.99$) is also reported.

Species assignment	Sampling Location	N	Country	*Alosa fallax*	*Alosa alosa*	Hybrids
Na	Poole Bay	9	England	9	0	0
	Severn	22	England	12	0	10
	Solway Firth	15	Scotland	4	3	8
	Tywi	13	Wales	4	0	9
	Tamar	60	England	0	25	35
Hybrid*	Minho	41	Portugal	3	33	5
*A. fallax**	Minho_SV	41	Portugal	30	0	11
*A. alosa**	Minho1	14	Portugal	0	12	2

*Populations from Sabatino *et al.,* 2021.

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Table 2: Populations description after hybrid identification and their removal (q = 0.99). Sample size (N), expected heterozygosity (H_E), observed heterozygosity H_O), number of alleles (N_A) and estimated fixation indices (F_{IS}) are detailed.

Species	Population	N	H_E	H_O	N_A	F_{IS}
A. fallax	Poole Bay	9	0.456	0.390	3.178	***0.241**
	Severn	12	0.487	0.504	3.588	0.058
	Minho_SV	30	0.508	0.474	3.706	***0.085**
A. alosa	Tamar	25	0.503	0.548	4.222	0.019
	Minho1	12	0.556	0.593	4.222	0.028

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