



## Short Communication

## A species-to-be? The genetic status and colonization history of the critically endangered Killarney shad

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## ARTICLE INFO

## Article history:

Received 7 April 2013

Revised 28 July 2013

Accepted 30 July 2013

Available online 8 August 2013

## Keywords:

*Alosa killarvensis*

Twaite shad

Speciation

Microsatellites

Control region

ABC

## ABSTRACT

Typically anadromous, the twaite shad (*Alosa fallax*) can become landlocked and adapt to a fully freshwater life. The only landlocked shad population in Northwestern Europe is found in a lake in Ireland, Lough Leane. The Killarney shad, *Alosa killarvensis* (or *Alosa fallax killarvensis*, as it is mostly referred to) displays a level of morphological divergence that indicates a long-term isolation in the lake. Microsatellites and mtDNA control region sequences were used within a coalescent framework (BEAST and Approximate Bayesian Computation (ABC)) to investigate its colonization history and to clarify its taxonomic status. Results indicate that the lake was likely colonized in two independent events, the first coinciding with the retreat of the ice sheet from the area after the Last Glacial Maximum and the second after the Younger Dryas. Microsatellite data has shown that these two landlocked lineages have completely admixed within the lake, and have started diverging significantly from their closest ancestor, the twaite shad. We argue that our molecular results (together with the life-history and physiological divergence between Killarney and twaite shad) are conspicuous enough to view the landlocked population as a new species, and one whose future existence would certainly not be insured by its sister taxon.

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## 1. Introduction

The twaite shad *Alosa fallax* (Lacépède 1803) and the allis shad *Alosa alosa* (L. 1758) are clupeids widely distributed along the North East Atlantic coasts, from Morocco to Iceland, and are among the rarest fish breeding in Ireland (King et al., 2011). They are typically anadromous, spending most of their life at sea and swimming back to the rivers to spawn. In certain conditions, by either natural or anthropogenic means, the link between the two habitats can become interrupted, and these fish then become trapped, or “landlocked”, in freshwater habitats, to which they can subsequently become fully adapted. Several examples of landlocked shad populations have been recorded across the species distribution range, mostly related to *A. fallax*, notably in the lakes of northern Italy and the Balkans (*Alosa agone* Scopoli 1786).

In North-western Europe, the only example of landlocked shad survives in the South West of Ireland, in Lough Leane, a 19 km<sup>2</sup> lake in Killarney National Park (Fig. S1). This putative species, ‘Killarney shad’ *Alosa killarvensis* (Regan 1916), is endemic to Ireland (Ferriss

et al., 2009). It is listed as ‘critically endangered’ by the IUCN Red List (Freyhof and Kottelat, 2008) and is threatened by eutrophication and invasive species (King et al., 2011). Nothing is known about how and when this twaite shad became landlocked: no anthropogenic barriers (weirs, locks) are in place in the river that connects Lough Leane with the sea and no adult migration has ever been recorded (Doherty et al., 2004). The lake is thought to be of glacial origin, forming approximately 16,000 years before present (BP; Jolly et al., 2012) with the retreat of the ice sheet (which covered the whole of the island during the Last Glacial Maximum (LGM); Clark et al., 2012). The origins of Ireland’s fauna and flora is still a contentious issue in biogeography; its geographical position as an outlying island in western Europe presents many difficulties in terms of how and when freshwater species in particular colonized it. Only Finnegan et al. (2013) thus far has attempted to date the origins of a fish species, the Atlantic salmon (*Salmo salar*), in Ireland. Clearly, a landlocked population can provide fascinating insights into the origin(s) of Irish freshwater fish, in terms of both timing and source populations.

The taxonomic status of this lacustrine shad has been a topic of debate for many years. Despite the species rank being the officially accepted name (Eschmeyer, 2013), the subspecies name, *Alosa fallax killarvensis*, remains the more widely adopted name in the

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literature. Compared to its anadromous counterpart, *Alosa fallax*, this 'dwarf' variant is characterized by a smaller body size and a lower raker count on the first gill arch (O'Maoileidigh et al., 1988; Doherty et al., 2004). Although early genetic investigations failed to detect any significant differentiation from *A. fallax* (O'Maoileidigh et al., 1988; Faria et al., 2006), a recent genetic study employing microsatellites (Jolly et al., 2012) unravelled strong genetic divergence between Killarney and anadromous twaite shad, and provided for the first time genetic evidence of the unique nature of this population. Traditionally employed phylogeographic markers such as the mtDNA cytochrome *b* and ND1 have been proven to have sufficient variability to distinguish between twaite and allis shad (Coscia et al., 2010; Jolly et al., 2012; Alexandrino et al., 2006; Faria et al., 2012) but are insufficient to investigate intraspecific variation (Faria et al., 2006). Therefore, more appropriate data and analyses are clearly needed to decipher the complex history of this landlocked shad. Here we couple the use of the non-coding, highly variable control region of the mtDNA with a suite of microsatellite markers, in order to (a) investigate the colonization process of Lough Leane (and, by reflection, of Irish freshwaters in a wider context) and (b) shed light on the taxonomic status of the Killarney shad.

## 2. Materials and methods

### 2.1. Data collection and handling

Samples of *Alosa killarneyensis* were collected in 2002 and 2003, as part of governmental monitoring surveys, using monofilament multi-mesh gill nets, whereas *A. alosa* and *A. fallax* were collected as by-catch of salmon netting in the lower end of Irish rivers. A total of 93 individuals were caught: 27 Killarney shad from Lough Leane, 34 twaite shad from the river Barrow and 32 allis shad from different estuaries in the south of Ireland (Fig. S1). Samples were identified by both gill-raker count and genetic screening (Coscia et al., 2010).

DNA was extracted following a standard salting-out procedure (Miller et al., 1988). Genus-specific primers were designed from the mitochondrial genome of *Alosa alosa* to amplify the whole control region (CR) of the mitochondrial DNA (mtDNA) (AloCR1-for, 5'-CAGTAGCTTAGTCTAAAGC and AloCR2-rev, 5'-GGCTTTCTACGACCCGTACT). DNA amplification was carried out in a final volume of 25  $\mu$ l, containing 22  $\mu$ l of Megamix Blue (Microzone Ltd.), 1  $\mu$ l of each primer (final concentration 0.4  $\mu$ M) and 25 ng of genomic DNA. Purified PCR products were sequenced by Macrogen Inc., assembled in Sequencher 4.7 (Gene Codes) in both directions, and manually aligned in Mega5 (Tamura et al., 2011). Amplification was successful in 41 out of 97 individuals (16 allis, 8 fallax and 27 Killarney shad). Eight microsatellites markers (Asa-2, Asa-6, Asa-8 and Asa-9; Waters et al. (2000); and Aa-14, Aa-20, Af-13 and Af-20; Faria et al. (2004)) were also amplified in two multiplex PCR reactions as described in Coscia et al. (2010), with all 97 individuals being successfully genotyped.

### 2.2. General statistical analyses

Phylogenetic trees were generated from the 41 sequences obtained (with no outgroup) with Bayesian genealogy sampling in BEAST v. 1.7 (Drummond et al., 2012). Times to most recent common ancestor (tMRCA) were estimated using five independent runs, each 10 million generations long with 10% burn-in, in order to avoid convergence issues. Amongst those available, three models were tested (constant population size, Yule, and Bayesian skyline plot) and Bayes factors were calculated in Tracer (Rambaut and Drummond, 2007). The Bayesian skyline plot was the best

supported model (constant population size, LnP = -1876.4; Yule, LnP = -1891.5; Bayesian skyline plot, LnP = -1875.7). A strict molecular clock and HKY mutation model (Hasegawa et al., 1985) were used, with a general divergence rate for fish mitochondrial control region of 11%/million years (Myr) (Patarnello et al., 2007). A median-joining network was constructed in Network 4.5 (Bandelt et al., 1999; www.fluxus-engineering.com) to explore the relationships among haplotypes.

Microsatellite genotypes were subjected to a test for the presence of null alleles, performed in FreeNA (Chapuis and Estoup, 2007).  $F_{ST}$  and  $F_{IS}$  values were calculated in Genetix 4.05 (Belkhir et al., 2004). The genetic relationships between species were visualized using a discriminant analysis of principal components (DAPC) from genotypic data, implemented in Adegenet (Jombart, 2008) for R (R Core Team, 2012).

### 2.3. Scenario reconstruction using Approximate Bayesian Computation

Approximate Bayesian Computation (ABC) was implemented in DIYABC v 1.0.4.46 (Cornuet et al., 2008) to further investigate the dynamics of the colonization process. In this software, microsatellites and sequence data can be combined in one analysis (Cornuet et al., 2010). However, in this study, no satisfactory statistical support was achieved by combining the two markers (data not shown), hence the analysis was repeated using the two classes of marker, separately.

First, using microsatellite data, we sought an independent estimate of the time since the split of the Killarney shad from its closest ancestor, as well as the effective population size of the extant populations. Although DIYABC is primarily designed to produce estimates of the relative posterior probabilities of alternative phylogeographic scenarios in a coalescent framework, here we had a relatively simplified situation, with three lineages whose evolutionary relationships are well characterized. We therefore compared the expected scenario, with twaite as the closest ancestor, with a counterintuitive one, assuming that Killarney originated from allis (Fig. S2A). Simulations were performed using the default parameters, with the only constraint being that the effective population size of Killarney could not be larger than either twaite or allis. Simulated data sets were created by requesting a total of 21 summary statistics, including number of alleles and heterozygosity (per population and per pairs of populations), Garza & Williamson's M-ratio (per population) and both  $F_{ST}$  and  $\delta\mu^2$  pairwise divergence statistics.

Since coalescent analysis performed in BEAST revealed the presence of two very distinct lineages within Lough Leane (see Results), we ran DIYABC with the mtDNA data to further clarify the process that brought about the presence of such clades in the lake. Seven scenarios were simulated, involving five populations representing the five major clades identified by the coalescent tree (Fig. 1A). Priors were as follows: effective population sizes for twaite and allis shad (N1, N4 and N5) between 10 and 100,000, for Killarney shad between 1 and 50,000 (N2, N3) and timing of events ( $t_0$ ,  $t_1$ ,  $t_2$ ,  $t_3$ ,  $t_4$ ) taken from the estimates from the coalescent tree. The most ancestral split between allis and twaite was fixed for all simulations at  $t_4$ , and the split between the two twaite clades (Twaite1 and Twaite2) at  $t_3$ . Scenarios were as follows: twaite shad could have entered the lake over two separate events, one clade at the time (at  $t_1$  and  $t_2$ , Scenarios 1, 2, 5, 6 and 7 Fig. S2B), or the lake could have been colonized once by a clade that then diverged in the lake (Scenario 4) or lastly, two clades entered the lake at the same time (Scenario 3). As recommended in Cornuet et al. (2010), scenarios including an unsampled lineage of different population size (N6, yellow in Fig. 3) were included in the analysis. All other parameters were set as default. Summary statistics

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