MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 1333–1346 doi: 10.1111/j.1365-294X.2011.05011.x

FROM THE COVER

All roads lead to home: panmixia of European eel in the Sargasso Sea

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Abstract

European eels (*Anguilla anguilla*) spawn in the remote Sargasso Sea in partial sympatry with American eels (*Anguilla rostrata*), and juveniles are transported more than 5000 km back to the European and North African coasts. The two species have been regarded as classic textbook examples of panmixia, each comprising a single, randomly mating population. However, several recent studies based on continental samples have found subtle, but significant, genetic differentiation, interpreted as geographical or temporal heterogeneity between samples. Moreover, European and American eels can hybridize, but hybrids have been observed almost exclusively in Iceland, suggesting hybridization in a specific region of the Sargasso Sea and subsequent nonrandom dispersal of larvae. Here, we report the first molecular population genetics study based on analysis of 21 microsatellite loci in larvae of both Atlantic eel species sampled directly in the spawning area, supplemented by analysis of European glass eel samples. Despite a clear East–West gradient in the overlapping distribution of the two species in the Sargasso Sea, we only observed a single putative hybrid, providing evidence against the hypothesis of a wide marine hybrid zone. Analyses of genetic differentiation, isolation by distance, isolation by time and assignment tests provided strong evidence for panmixia in both the Sargasso Sea and across all continental samples of European eel after accounting for the presence of sibs among newly hatched larvae. European eel has declined catastrophically, and our findings call for management of the species as a single unit, necessitating coordinated international conservation efforts.

Keywords: *Anguilla*, hybridization, individual assignment, isolation by distance, panmixia, spawning migration

Received 6 October 2010; revision received 12 December 2010; accepted 14 December 2010

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Introduction

Different species can exhibit widely different levels of genetic differentiation over a given spatial scale, depending on the vagility and life history of the organism and the environment it inhabits (Hamrick & Godt 1996; Waples 1998). However, even highly vagile organisms

such as many marine organisms persisting in environments without obvious barriers to gene flow typically exhibit low, but statistically significant genetic differentiation (Nielsen *et al.* 2009a). Testing correlation between environmental factors and genetic structure (landscape genetics) and analysing footprints of selection at the genomic level (population genomics) have furthermore led to a growing understanding that oceanographic features may shape the structure of populations of marine high-gene flow species (Jørgensen *et al.* 2005; Fontaine *et al.* 2007; Selkoe *et al.* 2010) and that local adaptation may occur (Nielsen *et al.* 2009b). Hence, it would be unexpected to find species that are widespread, are distributed across different environments and yet are truly panmictic.

The spawning biology and population genetic structure of the European eel (*Anguilla anguilla*) is a classical scientific mystery. Adult eels are distributed in freshwater and coastal regions, ranging from Subarctic environments in the Kola Peninsula and North Cape in northern Europe to subtropical environments in Morocco and the Mediterranean regions of Egypt (Tesch 2003). Yet, for centuries, the spawning places of European eel remained unknown. It was not until the early 20th century that Schmidt (1923) identified the southern Sargasso Sea as the spawning area of both European and American eels (*A. rostrata*). Since that time, spawning has been demonstrated to take place in frontal zones generated by the Subtropical Convergence, extending more than 1000 km from East to West (Kleckner & McCleave 1987) (Fig. 1). The larvae are subsequently transported by the Gulf Stream and other oceanic currents to the coastal and freshwater foraging areas in Europe/North Africa and North America, respectively, with European eel migrating a much longer distance (>5000 km) than American eel (~2000 km) (Tesch 2003).

The issue of panmixia vs. population genetic differentiation in European eel is highly controversial. Schmidt (1923) was able to separate European and American eels based on vertebrae or myomere counts but observed no geographical differentiation within species. Later studies using allozyme and mitochondrial DNA markers did not allow the rejection of the panmixia hypothesis in either species (Avise *et al.* 1986; Lintas *et al.* 1998; van Ginneken & Maes 2005). More recently, however, microsatellite marker studies of European eel (Daemen *et al.* 2001; Wirth & Bernatchez 2001) reported low, but significant, genetic differentiation and weak, but significant, isolation by distance, suggesting geographical genetic differentiation and providing evidence against panmixia. This conclusion was later questioned by studies that found differentiation between temporal samples of glass eels (metamorphosed juvenile eels) arriving at different times at the same locales, but not on a geographical scale (Dannewitz *et al.* 2005; Maes *et al.* 2006) or found slight temporal variation between cohorts of adult eels but no geographical differentiation (Palm *et al.* 2009). These observations suggest genetic differentiation caused by spawning time differences and a strong variance in reproductive success among individuals and spawning groups in the Sargasso Sea, leading to genetic differentiation between different 'arrival waves' of glass eels.

Resolving the panmixia controversy has become even more important because of recent drastic declines of the

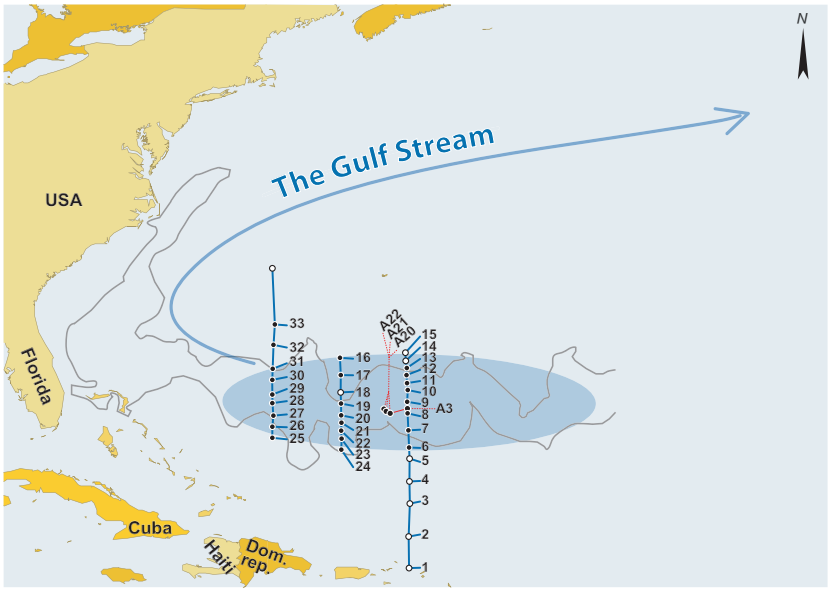

Fig. 1 Map showing the sampling stations in the Sargasso Sea. Stations marked by black dots indicate that European and/or American eel larvae were sampled, whereas white dots indicate that no eel larvae were sampled. The blue area outlines the approximate spawning area (Schmidt 1923; Tesch 2003), and the grey line indicates the Subtropical Convergence zone.

species. Recruitment has declined to <5% compared with pre-1980 levels, primarily because of over-exploitation and habitat destruction (van Ginneken & Maes 2005; Astrom & Dekker 2007). European eel is now listed as 'critically endangered' in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (http://www.redlist.org). However, it remains uncertain whether the species should be managed as one single panmictic unit or as several demographically independent populations.

Another unresolved issue concerns hybridization. European and American eels are known to hybridize, but hybrids are observed almost exclusively in Iceland (Avise *et al.* 1990; Albert *et al.* 2006). This observation could indicate the presence of a distinct hybrid zone in the Sargasso Sea, followed by nonrandom distribution of juvenile eels to coastal regions with hybrids predominantly being advected towards Iceland (van Ginneken & Maes 2005). Alternatively, if the two species exhibit different duration of the larval phase, then hybrids could exhibit an intermediate length larval phase, hence undergoing metamorphosis to glass eels in the ocean close to Iceland, situated between continental North America and Europe (Avise *et al.* 1990; Albert *et al.* 2006).

Investigation of both panmixia and hybridization has so far been based exclusively on continental samples of juvenile or adult eels. However, the reproducing unit is the appropriate unit for making inferences regarding the population structure of a species (Bowen *et al.* 2005; Carlsson *et al.* 2007). These issues prompt direct and thorough reassessments of the population structure and hybridization at the breeding location. Thus, we conducted a study based on samples taken directly in the spawning area in the Sargasso Sea, which are supplemented with samples of European glass eels from their major distributional range (from Iceland to Morocco).

Materials and methods

Sampling and molecular analyses

Eel larvae were sampled in the Sargasso Sea in March–April 2007 during the Danish Galathea 3 marine expedition. Sampling was conducted at 37 stations distributed across four separate transects, along the longitudes 64°W, 65°W, 67°W and 70°W (Fig. 1 and Fig. S1, Supporting information). For detailed sampling procedures, we refer to the study by Munk *et al.* (2010). In total, the samples covered 600 km of the Subtropical Convergence frontal zone in the Sargasso Sea, extending from East to West (Fig. 1). Larvae were sorted, measured to the nearest millimetre and stored in RNALater™ (Qiagen, Hilden, Germany) or 96% ethanol.

DNA was extracted (AllPrep™ DNA/RNA kit, Qiagen or E.Z.N.A.™ kit; Omega Biotek, Norcross, GA, USA) from the entire larva for molecular species identification and analysis of microsatellite markers. European eel, American eel and several species of non-anguillid eels spawn in the Sargasso Sea (Castonguay & McCleave 1987). Individual larvae were identified to species, i.e. European and American eels, based on analyses of the mitochondrial cytochrome *b* gene (Trautner 2006), the nuclear 5S rRNA gene (Pichiri *et al.* 2006), and genotype data for 21 microsatellite markers, applying Bayesian model-based clustering as implemented in STRUCTURE 2.3.1 (Pritchard *et al.* 2000). A total of 480 potential *Anguilla* larvae were sampled, among which 92 individuals either did not amplify for the three different types of markers or produced band or allelic patterns completely different from those expected for European and American eels. On closer inspection, these individuals typically exhibited morphological features (e.g. pigmentation) providing further evidence that they were larvae of non-anguillid eels. In total, 271 European and 117 American eel larvae were identified.

A total of 1010 European glass eels were sampled at fourteen geographical locations ranging from Iceland in the north to Morocco in the south. Some of these samples have also been analysed in other studies (Wirth & Bernatchez 2001; Maes *et al.* 2006), but based on different sets of microsatellite markers. From four of the locations, temporal samples were available (see Fig. 2 for sampling locations, sample sizes and sampling years). DNA was extracted using the E.Z.N.A.™ kit (Omega Biotek).

The samples of eel larvae and glass eels were genotyped using 21 microsatellite DNA markers: Ajms03, Ajms06 and Ajms10 (Tseng *et al.* 2001); Ajtr27, Ajtr37, Ajtr42 and Ajtr45 (Ishikawa *et al.* 2001); Ang101, Ang114 and Aro63 (Wirth & Bernatchez 2001); Aan01, Aan03 and Aan05 (Daemen *et al.* 1997); AangCA58, AangCT53, AangCT68, AangCT76, AangCT82, AangCT87, AangCT89 (Wielgoss *et al.* 2008); AjFABP, which is currently unpublished. The primers AjFABP-F: GGCAGTCG-ATGGAAACAAAC, AjFABP-R: CGTGTGCTCACAA CGTTACC were developed based on *Anguilla japonica* sequences of the heart fatty acid binding protein (H-FABP) gene (GenBank accession number AB039666.3). Markers were amplified in five multiplexes containing 3–8 loci using the Qiagen® PCR multiplexing kit according to the manufacturer's recommendations. We used 0.5 μL of DNA template in 13 μL reactions, an annealing temperature of 57 °C and 30 PCR cycles. Amplified fragments (0.5–0.8 μL) were added to 10 μL of Hi-Di Formamide and LIZ™ size standard solution (100:1) and analysed using an automated sequencer (ABI 3130 Genetic Analyser; Applied Biosystems, Foster City, CA,

Fig. 2 Map showing the sampling locations, sampling years and sample sizes of European glass eels.

USA). Genotypes were scored using GeneMapper™ version 4.0 (Applied Biosystems).

Analysis of genetic variation

For each microsatellite locus and within each sample, the expected and observed heterozygosity and number of alleles were estimated using Arlequin version 3.11 (Excoffier *et al.* 2005). Deviations from Hardy–Weinberg equilibrium because of heterozygote deficits were tested by permutation tests to determine if $F_{IS} > 0$, as implemented in FSTAT 2.9.3.2 (Goudet 1995).

Model-based clustering

Bayesian model-based clustering of multi-locus genotype data, as implemented in STRUCTURE 2.3.1 (Pritchard *et al.* 2000), was used for assigning individuals to clusters corresponding to species and for identifying potential hybrids. The analyses were conducted for two data sets: (i) one consisting of 388 European and American eel larvae from the spawning area and (ii) one containing all 388 eel larvae and all 1010 European glass eels. An admixture model was applied without using prior information on population structure and assuming that allele frequencies are independent. The number of clusters (k) with the highest posterior probability was identified using replicate runs assuming k from 1 to 10. A burn-in length of 100 000 steps was followed by 500 000 steps to obtain accurate parameter estimates, and 10 replicate analyses were conducted for each value of k to verify the consistency of the results. The most likely number of clusters was evaluated based on the posterior probability of k and the Δk method (Evanno *et al.* 2005).

Analysis of genetic population structure

The subsequent analyses were conducted for European eel only because of limited geographical sampling of American eel (see Fig. 1 and Fig. S1, Supporting information).

The statistical power to detect genetic heterogeneity at various true levels of differentiation was evaluated

for the present set of samples, number of loci and allele frequencies using POWSIM 4.0 (Ryman & Palm 2006). POWSIM simulates sampling of genes from a specified number of populations that have diverged because of random drift into an expected predefined level of differentiation (measured as F_{ST}). Samples were drawn from the simulated populations and used for testing genetic homogeneity using Fisher's exact test based on all loci simultaneously. Estimates of power were obtained as the proportion of significant outcomes when repeating the simulations 1000 times for each level of F_{ST}. POWSIM cannot handle markers with more than 50 alleles. Therefore, rare alleles were pooled for the few markers that exhibited more than 50 alleles (Table S1, Supporting information).

Global F_{ST} (Weir & Cockerham 1984) was computed and genetic differentiation among samples tested using the G-test (Goudet *et al.* 1996) as implemented in the ADEGENET 1.2-3 package (*R*) (Jombart 2008). Pairwise F_{ST} for different combinations of samples was estimated using the *Fstat* function of the GENELAND-package (*R*) (Guillot *et al.* 2005), which computes *F*-statistics using Weir & Cockerham's (1984) estimators. Estimation of two- and three-level hierarchical *F*-statistics was performed using the HIERFSTAT 0.04-4 (Goudet 2005) package in *R*. The significance of *F*-statistics was tested by permuting individuals 1000 times among samples and, for the hierarchical analyses, by permuting individuals among samples and samples among groups.

Isolation by distance was tested by pooling samples within transects, calculating pairwise values of $F_{ST}/(1-F_{ST})$ (Rousset 1997) and measuring East–West geographical distance between transects. The significance of the correlation coefficients between genetic and geographical distance was tested by permuting individuals among samples and estimating pairwise F_{ST} and r^2 20 000–50 000 times in *R*, and comparing observed values with the obtained null distribution of r^2 values.

Isolation by distance could be masked by different ages of larvae, where older larvae would be expected to have drifted longer from East to West along the thermal fronts (Fig. 1), whereas younger larvae would be sampled closer to where they were born. It is also possible that temporal reproductive isolation (isolation by time; Hendry & Day 2005) could be misidentified as isolation by distance, if different spawning events had occurred at random along the thermal fronts. We therefore tested for isolation by distance controlling for possible isolation by time, and for isolation by time controlling for possible isolation by distance. Specifically, we tested for correlations between pairwise individual genetic distances, geographical distances (latitudinal distance, longitudinal distance or total distance) and temporal 'distances' (i.e. differences of hatch date between individuals) using the partial Mantel test (Legendre & Legendre 1998) of the NCF package in *R*. *P*-values for the partial Mantel tests were based on 1000 permutations. Rousset's (2000) genetic distance between individuals was estimated using SPAGeDi 1.3 (Hardy & Vekemans 2002). Hatch date was calculated from capture date and length measurements (see Fig. S2, Supporting information) by assuming a hatch size of 3 mm and growth rate of 0.6 mm/day until a length of 8 mm, followed by a growth rate of 0.38 mm/day (personal communication: Jonna Tomkiewicz, National Institute of Aquatic Resources, Technical University of Denmark; Castonguay 1987; Kuroki *et al.* 2006). Some eel larvae were measured immediately after sorting and subsequently stored in RNAlater, whereas others were recovered from plankton samples stored in 96% ethanol for approximately 6 months before length measurements. Assuming that eel larvae shrink to approximately 85% of their original length when stored in ethanol (Peter Munk, National Institute of Aquatic Resources, Technical University of Denmark, personal observation), we increased the length measurements of these samples accordingly. Assuming different rates of shrinking (e.g. 90%) did not provide qualitatively different results.

To rule out the effect of sampling families, half-sibs and full-sibs within samples of eel larvae were identified by applying the maximum-likelihood method implemented in COLONY 2.0.0.1 (Wang 2004; Jones & Wang 2010). Pairs of individuals identified as sibs but sampled at different stations were considered unlikely to be real sibs, whereas individuals identified as sibs and sampled in the same haul were considered possible true sibs. The analysis was based on the full likelihood method and the 'long length of run' and 'high likelihood precision' options implemented in COLONY. Relevant statistical analyses (F_{ST}, isolation by distance and partial Mantel tests) were repeated by excluding one randomly chosen individual from each identified pair of half- or full-sibs, thereby investigating the effect of siblings.

Glass eels were sampled from the major distributional range of adult European eel, and all putative spawning populations of the European eel were probably represented in this sample. However, the possibility that the samples from the spawning area do not represent the entire eel population is a potential risk. To exclude the possibility of the presence of such unsampled 'ghost populations', we pooled all European eel larval samples from the Sargasso Sea and estimated the probability of assignment to this pooled sample for each individual glass eel. This analysis was conducted

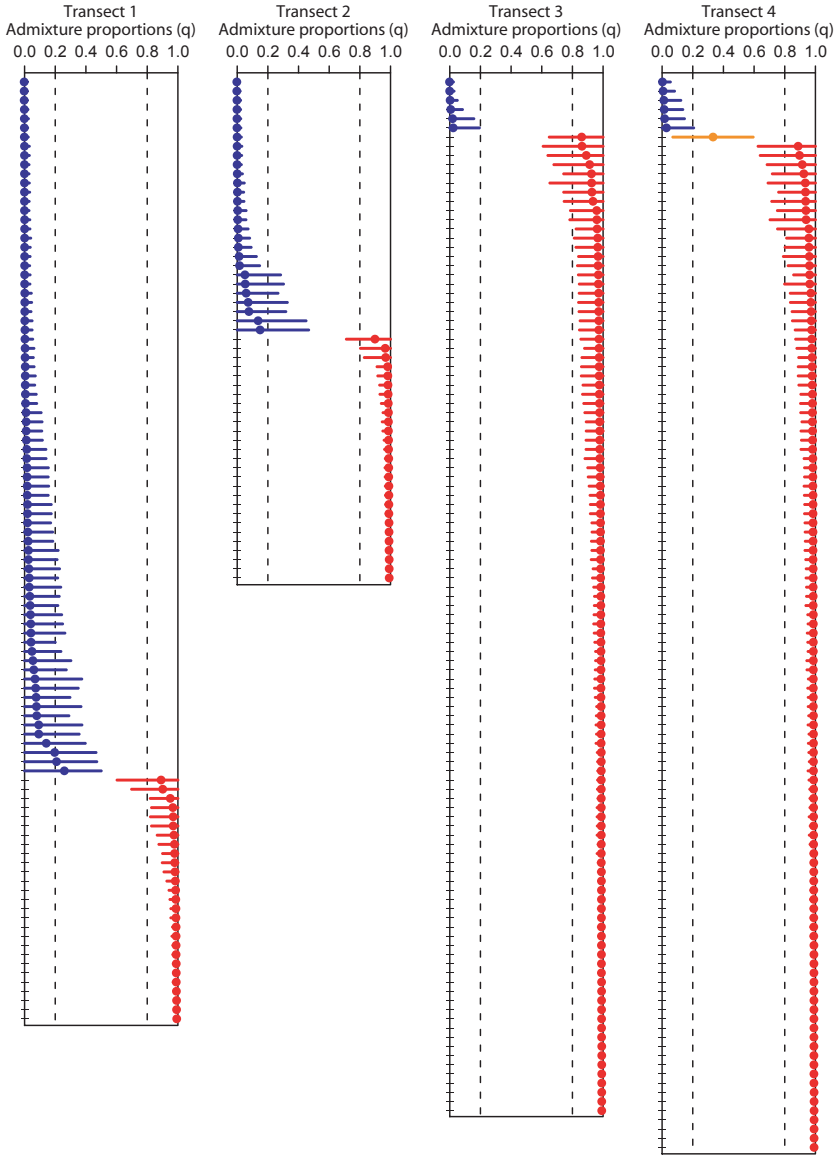

Fig. 3 Individual admixture proportions and their 90% confidence intervals for Sargasso Sea eel larvae sampled at each transect, estimated using STRUCTURE (Pritchard *et al.* 2000) and assuming the presence of two clusters corresponding to European and American eels (*q* denotes the probability of belonging to the European eel cluster). Values for American eel are presented in blue, for European eel are shown in red, and a single putative hybrid is indicated by orange.

using the simulation test by Paetkau *et al.* (2004) based on 100 000 simulated individuals, as implemented in GENECLASS 2 (Piry *et al.* 2004).

Results

Distribution of European and American eel larvae

The geographical distribution of the sampled 271 European and 117 American eel larvae showed a predominance of American eel in the West, gradually replaced by European eel towards the East (Fig. S1, Supporting information and Fig. 3). The North–South distribution of individuals across transects clearly showed a concentration of larvae in the main frontal zone, confirming that larval recruitment is associated with thermal fronts (Kleckner & McCleave 1987) (Fig. S1, Supporting information). Therefore, genetic differentiation would be expected to occur along an East–West axis and we pooled samples into four transect samples. These four transect samples formed the basis of all subsequent analyses based on Sargasso Sea eel larvae. However, the alternative grouping along the North–South axis was also examined.

Analysis of genetic variation

Number of alleles, observed and expected heterozygosity for each of the 21 microsatellite loci in each sample are shown in Table S1, Supporting information. Tests for Hardy–Weinberg equilibrium based on F_{IS} estimates yielded 11 significant outcomes in European eel and four significant outcomes in American eel among 608 tests after false discovery rate correction (Benjamini & Yekutieli 2001) (Table S2, Supporting information). Except for four significant outcomes among 25 tests involving Ajms10 in European eel and two significant outcomes among four tests involving AangCA58 in American eel, there were no general tendencies towards heterozygote deficits at the loci.

Model-based clustering and hybridization

Based on the analysis of microsatellite markers, significant genetic differentiation was found between European and American eel larvae ($F_{ST} = 0.09$, $P < 0.001$), suggesting sufficient statistical power for detecting hybrids. STRUCTURE (Pritchard *et al.* 2000) analyses of (i) the Sargasso Sea eel larvae and (ii) the combined data set of eel larvae and European glass eels in both cases identified $k = 2$ as the most likely number of clusters, corresponding to the two species (data not shown). Subsequent analysis of the European eel samples suggested $k = 1$, thus providing no evidence for intraspecific genetic differentiation (data not shown).

Obtained q-values (individual admixture proportions) with corresponding 90% confidence intervals (CI) were inspected, and individuals with q-values close to 0 and lower 90% confidence limits overlapping with 0 were identified as American eel, whereas individuals with q-values close to 1 and upper 90% CI encompassing 1.0 were identified as European eel. Admixed individuals (i.e. with intermediate q-values) for which the 90% CI contained neither 0.0 nor 1.0 were considered to be hybrids between the two species. Individual admixture proportions for the Sargasso Sea eel larvae showed that the two species could be clearly separated (Fig. 3). Only a single individual showed intermediate admixture proportions ($q = 0.338$, $CI_{90}\% = 0.070–0.594$), suggesting that this was a hybrid. Among the 1010 European glass eels, three putative hybrids were identified, including one individual from Iceland (Fig. 4).

Genetic differentiation among European eel larvae

Larger and thus older eel larvae may have been born a considerable distance from where they were sampled. Therefore, we analysed genetic differentiation using only individuals measuring <15 mm ($n = 220$; see Fig. S2, Supporting information). An analysis of statistical power using POWSIM (Ryman & Palm 2006) showed that the sample sizes and specific genetic markers applied were adequate for detecting a low level of genetic differentiation ($F_{ST} = 0.0011$) with a high probability (>0.9) (Fig. 5).

Very low and nonsignificant genetic differentiation was observed among the four transect samples ($F_{ST} = 0.00076$, $P = 0.0870$, $n = 220$), and pairwise F_{ST} values were also low and nonsignificant (Table S3, Supporting information). Significant correlation ($r^2 = 0.8175$, $P = 0.0098$) between genetic ($F_{ST}/1-F_{ST}$) and geographical distance was nevertheless detected (Fig. 6a), potentially indicating East–West isolation by distance.

Sampling larvae only a few days old has a risk of including sibs within the same hauls, which could create subtle, but significant, correlations. The method for reconstructing sibships implemented in COLONY (Jones & Wang 2010) identified two full-sib families at two stations in the two westernmost transects, comprising three and two full-sibs, respectively (Fig. 7). Twenty-two pairs of putative half-sibs were additionally identified within stations. It must be considered highly unlikely that individuals collected at different stations are true sibs. Hence, comparing the number of putative sibs within stations with the number of putative sibs sampled at different stations provides an indication of the accuracy of the COLONY analysis. No full-sib pairs were identified involving individuals from different stations, and Fisher's exact test comparing observed and expected numbers of full-sib pairs yielded a highly significant outcome ($P = 0.00008$). In contrast, 247 possible half-sib pairs were identified between stations, and the number of half-sib pairs within and between stations was not significantly different (Fisher's exact test, $P = 0.6041$). Hence, the identified full-sib families are likely to represent real sibs, whereas several of the putative half-sib pairs are likely to represent false positives.

Random removal of all but one individual from each pair of full-and half-sibs identified within stations substantially changed the results. Genetic differentiation among the four transects decreased further ($F_{ST} = -0.000287$, $P = 0.4825$, $n = 199$), and the apparent isolation by distance became nonsignificant ($r^2 = 0.1804$, $P = 0.4739$; see Fig. 6a). As half-sibs are likely to include false positives, we further repeated the analysis by only excluding one individual of each full-sib pair. Again, this yielded low and nonsignificant genetic differentiation ($F_{ST} = 0.00019$, $P = 0.4755$, $n = 217$) and nonsignificant isolation by distance ($r^2 = 0.4728$, $P = 0.1475$). Finally, to further verify that the apparent isolation by distance could be explained solely by

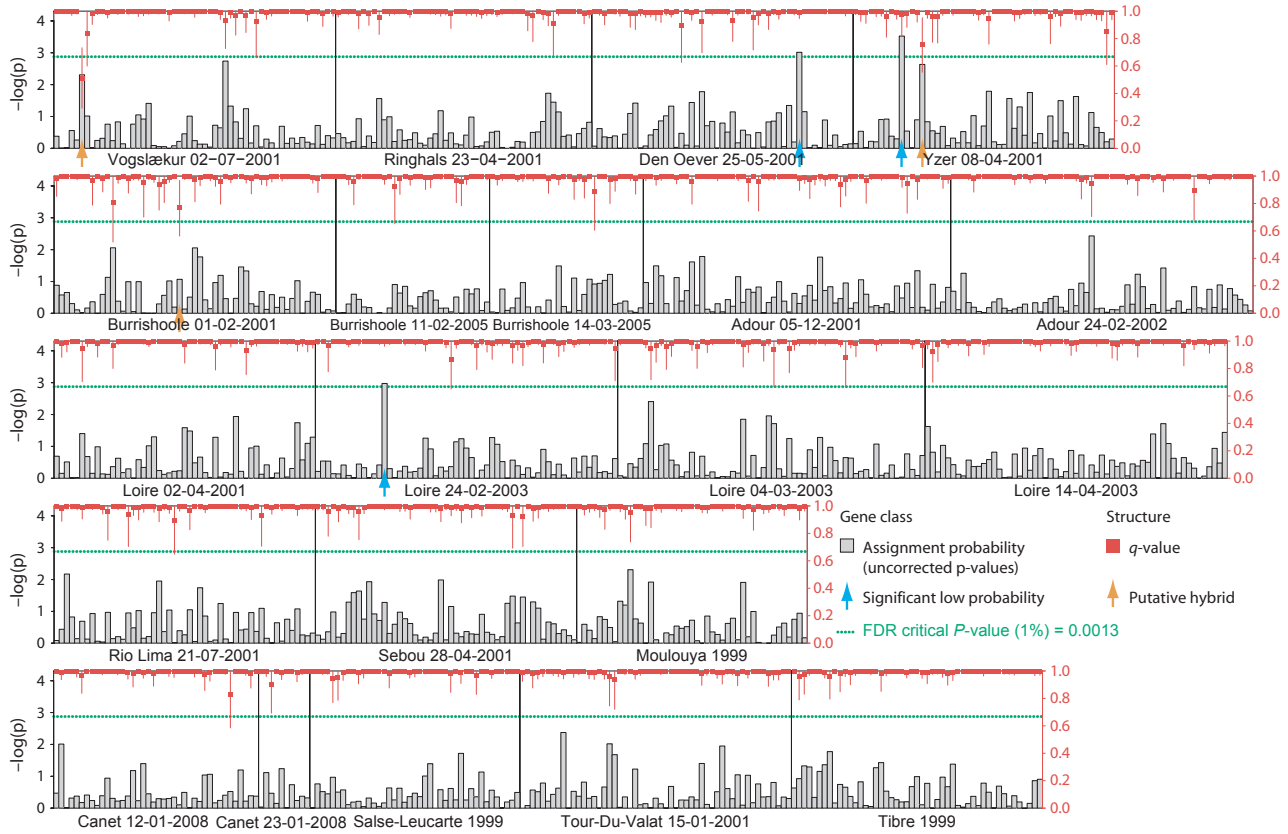

Fig. 4 Individual admixture proportions and assignment probabilities for individual glass eels from Europe and North Africa. Individual admixture proportions (q) with corresponding 90% confidence intervals, estimated using STRUCTURE (Pritchard *et al.* 2000), are shown in red for each individual. The analysis assumes $k = 2$ corresponding to European and American eels, and q denotes the probability of belonging to the European eel cluster. Bars denote the probability of assigning an individual glass eel to the pooled sample of European eel larvae from the Sargasso Sea, using a simulation test implemented in GENECLASS2 (Piry *et al.* 2004). The green dotted line denotes the false discovery rate-corrected (Benjamini & Yekutieli 2001) critical P-value at the 1% significance level. Note that the scale on the left y-axis represents $-\log_{10}(P)$, and a high value corresponds to a low probability.

sampling of two full-sib families, we randomly removed three unrelated individuals from the transect samples where the sibs were identified, analysed isolation by distance and repeated this procedure 1000 times. The fitted isolation-by-distance line was very similar to that obtained when full-sibs were included, and the 95% confidence interval of simulated r^2 values exceeded the r^2 value observed when full-sibs were removed (Fig. 6b). Hence, we conclude that the apparent isolation by distance represents artefacts caused by inclusion of only a few sibs.

We further investigated if different groupings of samples, e.g. by pooling stations into four arbitrary groups from north to south, or different thresholds for size of larvae included in the analyses could affect the results. In none of the cases, significant F_{ST} or isolation by distance was observed (Table S4, Supporting information). Hierarchical analysis of genetic differentiation (Goudet 2005) also yielded low and nonsignificant differentiation among transects and among stations within transects (Table 1).

Some previous studies suggested that genetic differentiation in European eel reflects isolation by time (Hendry & Day 2005) rather than distance (Dannewitz *et al.* 2005; Maes *et al.* 2006). We therefore conducted partial Mantel tests (Legendre & Legendre 1998) analysing the association between pairwise individual genetic distance (Rousset 2000), geographical distance and 'temporal distance' (differences in estimated hatch date between individuals). However, all associations were nonsignificant whether or not the effect of sibs within samples was removed, and whether or not longitudinal, latitudinal or total geographical distances between individuals were considered (Table S5, Supporting information).

In total, the analysis of samples from the Sargasso Sea yielded no evidence for genetic differentiation, isolation by distance or isolation by time.

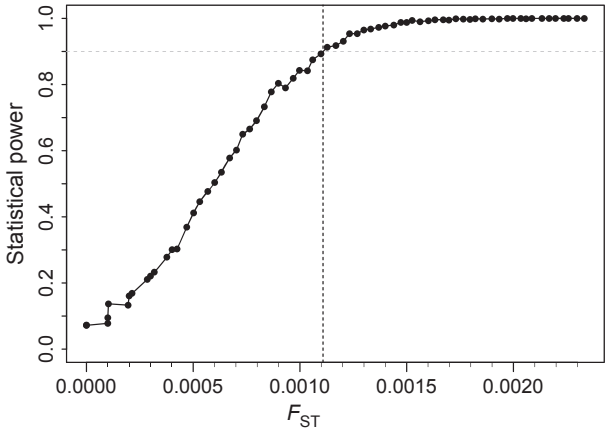

Fig. 5 Simulated statistical power for obtaining significant outcomes in tests of genetic differentiation involving four samples and the specific marker characteristics and sample sizes of Sargasso Sea European eel larvae. Simulations were performed using POWSIM 4.0 (Ryman & Palm 2006). The dotted line indicates the minimum level of genetic differentiation that can be detected with 90% statistical power.

Genetic differentiation among glass eels

Nonsignificant genetic differentiation was found among the total of 21 geographical and temporal samples of glass eels (F_{ST} = 0.00024, P = 0.2464), and there was no evidence for isolation by distance (r^2 = 0.0008, P = 0.9283; see Fig. 6c). Constraining the analysis to the year with most samples (2001) also showed nonsignificant genetic differentiation (F_{ST} = 0.00021, P = 0.5345) and no evidence for isolation by distance (r^2 = 0.0640, P = 0.3612; see Fig. 6c). Three-level hierarchical F-statistics (Goudet 2005) provided no evidence for temporal genetic differentiation between glass eels sampled in different years from the same site or between glass eels sampled on different dates within the same year and site (Table 1).

Hence, similar to the analyses of Sargasso Sea eel larvae, we found no evidence for genetic differentiation, isolation by distance or isolation by time in continental samples of European glass eels.

Relationships between Sargasso Sea eel larvae and continental glass eels

No significant genetic differentiation was observed between the pooled sample of European eel larvae from the spawning area and glass eels from Europe and North Africa (F_{ST} = −0.00012, P = 0.2136, n = 1282). To fully confirm that the samples of glass eels and eel larvae belonged to the same panmictic population, and to exclude the possibility of unsampled 'ghost populations', the probability of assigning each individual glass

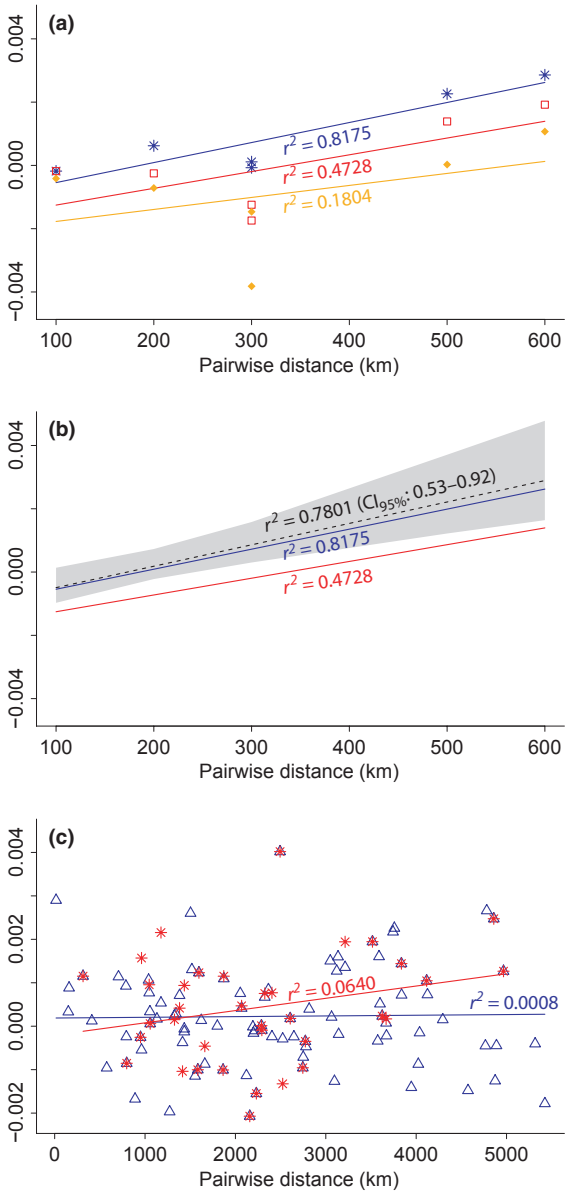

Fig. 6 Analyses of isolation by distance based on correlating genetic (F_{ST}/1−F_{ST}) vs. geographical distance. (a) European eel larvae <15 mm. Results including putative half- and full-sibs are shown in blue (r^2 = 0.8175, P = 0.0098), results obtained when excluding one individual from each putative full- or half-sib pair are shown in orange (r^2 = 0.1804, P = 0.4739) and results obtained when excluding one individual from each full-sib pair are shown in red (r^2 = 0.4728, P = 0.1475). (b) The mean isolation-by-distance line generated by randomly excluding three nonfull-sib individuals 1000 times (r^2 = 0.7801, CI_{95}% = 0.53; 0.92) is shown in black. The shaded area represents 95% confidence limits of the black line. The isolation by distance lines including putative half-and full-sibs (blue line) and excluding one individual from each full-sib pair (red line) have been added for comparison. (c) Continental samples of glass eels. Results involving all samples are shown in blue (r^2 = 0.0008, P = 0.9283), and results encompassing only samples from 2001 are shown in red (r^2 = 0.0640, P = 0.3612).

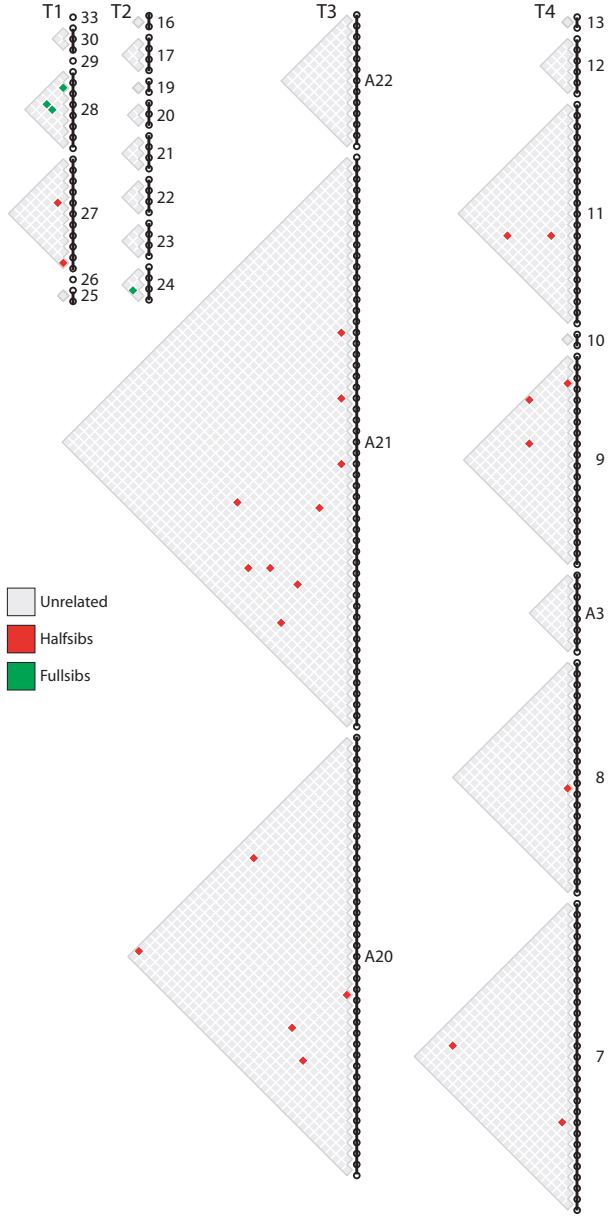

Fig. 7 Pairs of putative full-sibs (green) and half-sibs (red) identified within stations using COLONY 2.0.0.1 (Jones & Wang 2010). Black dots denote individual eel larvae, and individuals from the same stations are connected by lines.

eel to the pooled sample of European eel larvae from the spawning area was estimated. Only three individuals (~0.3%) showed significantly low *P*-values (at the 1% level) after false discovery rate correction (Benjamini & Yekutieli 2001) (Fig. 4). Individual admixture proportions estimated using STRUCTURE (Pritchard *et al.* 2000) did not suggest that any of these individuals were hybrids between European and American eels, although three other putative hybrids were observed (Fig. 4). No geographical or temporal pattern was found in the occurrence of glass eels with significantly low assignment probabilities and, in total, the presence of unsampled populations not represented by the sample from the spawning area appears unlikely.

Table 1 Hierarchical *F*-statistics for eel larvae and glass eel samples, as implemented in HIERFSTAT 0.04-4 (Goudet 2005). All *P*-values are based on 1000 permutations

Data set		*F*-value	*P*-value
Larvae < 15 mm excl. sibs* (n = 188)			
Among transects	F_{CT}	−0.00034	0.218
Among stations within transect	F_{SC}	0.00223	0.506
Larvae < 15 mm excl. full-sibs* (n = 206)			
Among transects	F_{CT}	−0.00011	0.3772
Among stations within transect	F_{SC}	0.00282	0.3639
Glass eels all years† (n = 1010)			
Among sites	F_{CT}	−0.00024	0.428
Among years within sites	F_{SC}	0.00158	0.072
Among dates within years	F_{ZS}	−0.00112	0.349
Glass eels† (n = 463)			
Among sites	F_{CT}	−0.00022	0.456
Among years within sites	F_{SC}	0.00146	0.110
Among dates within years	F_{ZS}	−0.00099	0.156

*One individual from each half- or full-sib pair was excluded, and stations with only one individual were excluded.
†The first data set consists of all samples, whereas the second only includes sites sampled at more than one time point: Adour (France), Loire (France) and Burrishoole (Ireland); temporal samples from Canet were not considered because of low sample size.

Discussion

The results of the study provide important information pertaining to the interpretation of findings of European × American eel hybrids and for resolving the controversy of panmixia in European eel. We discuss these issues in detail in the following section.

Hybridization between European and American eels

Our study documents that the spawning areas of the two species show considerable geographical overlap, and ample opportunities for hybridization exist. Nonetheless, the finding of only a single putative hybrid does not support the presence of a distinct marine hybrid zone. The occurrence of hybrid eels in Iceland (Avise *et al.* 1990; Albert *et al.* 2006) is unlikely to reflect nonrandom advection of hybrid eel larvae from a specific region of the Sargasso Sea (van Ginneken & Maes 2005). However, it does not contradict the alternative hypothesis that larval phase duration is intermediate in hybrids when compared to the parental species (Avise *et al.* 1990; Albert *et al.* 2006). Although low in frequency (<1%), hybrids will be numerically important

because of the abundance and high fecundity of the species. This could lead to metamorphosis and settlement of substantial numbers of hybrid eel larvae halfway on the route towards Europe, corresponding to the geographical location of Iceland.

Panmixia in European eel

This study represents the most comprehensive European eel data set to date and includes unique samples of larvae from the spawning area, along with glass eel samples from continental foraging areas. The results offer new and decisive insights into the European eel panmixia controversy (Daemen *et al.* 2001; Wirth & Bernatchez 2001; Dannewitz *et al.* 2005; Maes *et al.* 2006; Palm *et al.* 2009). All results are in accordance with a panmixia scenario. Despite considerable statistical power, there was no evidence for genetic differentiation among samples of larvae from the Sargasso Sea. Although weak but significant isolation by distance was observed, it was demonstrated that this was an artefact caused by inclusion of a few sibs in some of the samples. This illustrates the inherent problems when interpreting very low genetic differentiation as often observed in marine organisms (Waples 1998); even small bias and artefacts may substantially affect results. Also, there was no evidence for more subtle structure caused by spawning time differences leading to isolation by time (Hendry & Day 2005).

The analysis of newly spawned eel larvae sampled directly in the spawning area represents an optimal strategy for testing the panmixia hypothesis, provided that the entire European eel population is represented in the analysed samples. To ensure that no unsampled populations exist, and to further test the panmixia hypothesis, we genotyped the same microsatellite markers in glass eels sampled on various dates and from sites ranging from Iceland to Morocco. Again, very low and nonsignificant genetic differentiation was observed and there was no evidence for isolation by distance. These results conflict with previous studies (Daemen *et al.* 2001; Wirth & Bernatchez 2001), potentially because of temporal variation in the degree of isolation by distance between years, and genetic diversity may have been lost during the last 30 years of declining eel population size (Astrom & Dekker 2007). However, three-level hierarchical analysis of genetic differentiation provided no evidence for temporal genetic differentiation between eels sampled in different years from the same site or between eels sampled on different dates within the same year and site. Thus, in accordance with a recent study of adult eels (Palm *et al.* 2009), we found no evidence for geographical or temporal differences among samples of glass eels in continental waters.

Finally, genetic differentiation between Sargasso Sea eel larvae and continental glass eel samples was very low and nonsignificant. Individual assignment of glass eels to the total European eel larvae sample showed that eels from the whole distributional range from Northern Europe to North Africa could be accounted for by spawning in the Sargasso Sea and that no unsampled 'ghost' populations were likely to exist.

The results suggest a random arrival of adult eels in the spawning area and subsequent random distribution of larvae across the European and North African coasts. This is in agreement with several biological features of the species. First, eel larvae are transported >5000 km by the Gulf Stream and other currents before reaching Europe and North Africa (Tesch 2003). This provides ample opportunities for mixing of aggregations of larvae from different parts of the spawning region, although oceanographic modelling has suggested that retainment of larval aggregations may be possible under some conditions (Kettle & Haines 2006). Second, the early life of eel larvae is intimately linked to the thermal fronts of the southern Sargasso Sea, and spawning must be assumed to take place in the frontal zones (Kleckner & McCleave 1987; Munk *et al.* 2010). The fronts are highly dynamic and undulate along an East–West axis (Tesch 2003; Munk *et al.* 2010). Precise homing of eels to their specific natal site in the Sargasso Sea therefore seems unlikely as this would conflict with the necessity of spawning in the thermal fronts. Moreover, data on the spawning migration of European eel (Aarestrup *et al.* 2009) and on the recent discovery of spawning individuals of the related species *A. japonica* and *A. marmorata* in the Pacific Ocean (Chow *et al.* 2009) suggest that both migration and spawning occur in the upper pelagic zone (<1000 m). Hence, imprinting and homing to specific bottom structures in the up to 5-km-deep Sargasso Sea appear unlikely. In total, we propose that spawning European eels exhibit a crude homing behaviour towards the frontal zone region of the southern Sargasso Sea, but fine-scale homing towards their precise natal sites does not take place.

The finding of panmixia in such a widely distributed species inhabiting environments ranging from Subarctic to Subtropical climates must be considered highly unusual. In many organisms, genotype × environment interaction and selection over this geographical and environmental scale would be expected to lead to local adaptation (Kawecki & Ebert 2004). However, local selection occurring across the continental distributional range within a generation would be obliterated in the 'genetic melting-pot' of the Sargasso Sea. Indeed, within-generation local selection acting on genes in linkage disequilibrium with studied genetic markers could

potentially explain the weak clinal patterns interpreted as genetic structure in previous studies (Daemen *et al.* 2001; Wirth & Bernatchez 2001). This issue could be further clarified by population genomics analyses (Luikart *et al.* 2003) aimed at identifying genes under selection in continental and Sargasso Sea samples.

Our findings have crucial implications for conservation of this endangered species. The recovery time of the species is expected to be very slow; ca. 80 years even in the case of complete closure of fisheries (Astrom & Dekker 2007), stressing the need for imminent action. Panmixia within the species implies that management and conservation efforts must be coordinated at the transnational level, as over-exploitation in one region will negatively affect the total European eel population and decrease recruitment across the whole distributional range.

Acknowledgements

We thank the Captains and crews of HMS Vædderen and the Newfoundland Alert for assistance with sampling, Françoise Daverat, Javier Lobón, Stefan Palm, Russell Poole, Alan Walker, and Håkan Wickström for providing glass eels, Karen-Lise D Mensberg for help in the laboratory and Sebastién Moore and two anonymous reviewers for constructive comments on the manuscript. The study was funded by the Villum Kann Rasmussen Foundation, Elisabeth and Knud Petersens Foundation, the European Union FP7 (EELIAD grant 212133), the Danish Council for Independent Research | Natural Sciences (grant 09-072120), and the Danish Expedition Foundation. The present work was carried out as part of the Galathea 3 expedition under the auspices of the Danish Expedition Foundation. This is contribution no. P72 of the Danish Galathea 3 expedition.

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The authors of this paper represent a multidisciplinary team of scientists with research interests spanning from evolutionary biology and population genomics to fish biology and biological oceanography. The research was undertaken as part of the Danish Galathea 3 marine expedition, which involved sampling of European and American eel larvae and oceanographical analyses in the Sargasso Sea during spring 2007. In addition to analyzing the genetic population structure of European eel the research team has studied the spawning migration of adult eels using satellite data storage tags, the feeding

biology of eel larvae using DNA barcoding of gut contents and the importance of oceanic fronts in the Sargasso Sea for the earliest life stages of eels.

Data accessibility

Data deposited at Dryad: doi:10.5061/dryad.8155.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Genetic variation at the 21 microsatellite markers for each of the 25 samples of larvae and glass eels of European and four samples of American eel larvae.

Table S2 F_{IS} estimates and tests for Hardy–Weinberg Equilibrium for each marker in each sample.

Table S3 Pair-wise F_{ST} between all pair-wise combinations of samples of European eel.

Table S4 Global F_{ST} values and corresponding correlation coefficients (r^2) for genetic [$F_{ST}/(1-F_{ST})$] vs. geographic distance.

Table S5 Partial Mantel tests of correlations between individual genetic distances and geographic and temporal distances (hatch date).

Fig. S1 Bar plots of the number of American and European eel larvae sampled at each station in the Sargasso Sea.

Fig. S2 Length distributions for the analyzed American and European eel.

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