

Natural hybrids in Atlantic eels (*Anguilla anguilla*, *A. rostrata*): evidence for successful reproduction and fluctuating abundance in space and time

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Abstract

The outcome of natural hybridization is highly variable and depends on the nonexclusive effects of both pre- and post-mating reproductive barriers. The objective of this study was to address three specific questions regarding the dynamics of hybridization between the American and European eels (*Anguilla rostrata* and *Anguilla anguilla*). Using 373 AFLP loci, 1127 eels were genotyped, representing different life stages from both continents, as well as multiple Icelandic locations. We first evaluated the extent of hybridization and tested for the occurrence of hybrids beyond the first generation. Second, we tested whether hybrids were randomly distributed across continents and among Icelandic sampling sites. Third, we tested for a difference in the proportion of hybrids between glass eel and yellow eel stages in Iceland. Our results provided evidence for (i) an overall hybrid proportion of 15.5% in Iceland, with values ranging from 6.7% to 100% depending on life stages and locations; (ii) the existence of hybrids beyond the first generation; (iii) a nonrandom geographic distribution of hybrids in the North Atlantic; and (iv) a higher proportion of first and later generation hybrids in yellow eels compared to glass eels, as well as a significant latitudinal gradient in the proportion of hybrids in Icelandic freshwater. We propose that the combined effect of both differential survival of hybrids and variation in hybridization rate through time best explain these patterns. We discuss the possibility that climate change, which is impacting many environmental features in the North Atlantic, may have a determinant effect on the outcome of natural hybridization in Atlantic eels.

Keywords: AFLP, *Anguilla*, climate changes, fitness, hybrid zone, hybridization

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Introduction

It is increasingly accepted that aside from the development of stable hybrid zones (Barton & Hewitt 1985), interbreeding between distinct species or populations can result in a variety of evolutionary outcomes, including reinforcement (*sensu* Dobzhansky 1937), genetic extinction (Rhymer & Simberloff 1996; Huxel 1999), speciation (Templeton 1981), enhanced genetic diversity (Rieseberg *et al.* 1999), and novel genetic combinations (Anderson & Stebbins 1954; Lewontin & Birch 1966; Arnold 2004; Seehausen 2004). Moreover, the viewpoint that natural hybridization may play a significant role in animal evolution has been revived

in recent years (Arnold 1992, 1997, 2004; Dowling & Secor 1997; Barton 2001; Mallet 2005).

The resulting outcome of natural hybridization is highly variable and depends on the nonexclusive premating and postmating reproductive barriers (Aldridge 2005). Postmating isolation has been discussed in the recent debate on hybrids' fitness relative to the parental forms in the wild and the evolutionary potential of natural hybridization (Arnold 1997). Hybrid fitness may vary greatly and is inversely proportional to the strength of the barriers. This variability is explained in part by the heterogeneity in space and time of endogenous as well as exogenous postmating selection pressures (e.g. Grant & Grant 1996), which in turn determine the fate of hybrids relative to the parental forms with which they coexist in a particular environment. Superior hybrid fitness may be due to

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heterosis via heterozygote advantage, silencing of deleterious recessive alleles or epistatic interactions, while reduced hybrid fitness may be caused by the disruption of co-adapted gene complexes. Although heterosis may prevail in the first hybrid generation, hybrid fitness may vary between hybrid classes (Arnold & Hodges 1995) as outbreeding depression accompanied by reduced fitness may occur in subsequent generations (e.g. Edmands 1999; Gharrett *et al.* 1999). While variation in the relative fitness of hybrids has been observed (Arnold & Hodges 1995; Burke & Arnold 2001) and theoretically predicted (Barton 2001), hybrid superiority relative to at least one of the parental forms is increasingly being documented (reviewed in Arnold 1997; Arnold *et al.* 1999).

In controlling the number of hybrids produced, premating reproductive barriers may as well play an important role in determining the genotypic composition and fate of a hybrid zone (Aldridge 2005). These barriers may be of different sources such as spatial, seasonal or behavioural isolation. For instance, the level of reproductive isolation might result from spatial and/or temporal overlap during reproduction periods (e.g. Emelianov *et al.* 2001; Bailey *et al.* 2004). Environmental factors might as well influence the level of hybridization by reducing the relative reproductive success for one gender in one of the parental form (e.g. Williams *et al.* 2001). Disturbance dynamics which often break down the isolating barriers might as well affect the level of gene flow in a specific habitat (e.g. Bleeker & Hurka 2001). Being affected by local ecological conditions, it is the strength and potentially the interaction of isolating barriers that will determine the extent of hybridization between two parental forms. It is therefore necessary to consider potential factors related to both pre- and post-mating barriers that will restrict contacts between the two hybridizing species or lineages in order to fully understand the role of natural hybridization in evolution (Bailey *et al.* 2004).

In this study, we document the dynamics of introgressive hybridization between the American and European eels (*Anguilla rostrata* and *Anguilla anguilla*). The life cycle of the two species begins in the Sargasso Sea (North Atlantic Ocean, 19.5–30°N, 48–79°W) where the larvae, called leptocephali, appear at the surface. Leptocephali then passively and perhaps actively follow the Gulf Stream and the North Atlantic drift to reach their respective continent: North America for the American eels and Europe for the European eels. This journey is longer for European compared to American eels and may last from 7 months to up to 3 years depending on authors (reviewed in Arai *et al.* 2000). Once larvae have reached a minimal threshold size, they undergo metamorphosis into glass eel (Otake 2003). The glass eel actively swims towards shore and gradually develops pigmentation. This life stage may last from 20 days to more than 160 days, depending on water temperature

and to a lesser extent on salinity conditions (Briand *et al.* 2005; Linton *et al.* in press). Once glass eels have lost their transparency, they are called elvers and later on yellow eels. Yellow eels are able to adapt and exploit almost all aquatic habitats, from coastal environments to headwaters. Feunteun *et al.* (2003) identified four different movement behaviours of eels during their yellow eel stage: founders, pioneers, home-range dwellers and nomads. All those behaviours represent different life strategies towards reaching a minimal threshold size before migrating back to the spawning grounds. After 3 to 20 years spent in their feeding grounds as yellow eels (Aoyama & Miller 2003), at the onset of maturation, they metamorphose into silver eels and begin their migration to the Sargasso Sea, where they reproduce and die.

Spawning grounds of both species overlap in space and time (McCleave *et al.* 1987), thus setting the stage for interbreeding. Although their biological species status is not debated anymore, Atlantic eels differ only slightly in morphology (Avisé 2003), vertebrae count being the only quasi-diagnostic phenotypic character (Boëtius 1980). In North America and continental Europe, eels rarely present ambiguous vertebrae counts. However, the occurrence of eels with American-like number of vertebrae in northern Europe, and particularly in Iceland has been pointed out previously (Boëtius 1980; Williams *et al.* 1984; Avisé *et al.* 1990). Genetic analyses indicated that these eels were most likely of hybrid ancestry (Avisé *et al.* 1990), although the markers used did not allow clarification of their hybrid status (e.g. first or later generation hybrids). This was due to the nondiagnostic nature of the allozyme loci used (*Mdh-2*), and the maternal inheritance of mitochondrial DNA (Avisé *et al.* 1990). In a recent study that aimed to clarify the status of ambiguous Icelandic individuals using microsatellite markers, Mank & Avisé (2003) concluded that 'available microsatellite data are inadequate to critically test [the] hypothesis... that some Icelandic eels are of hybrid ancestry'. Sample size and geographic coverage of Icelandic locations were also both very limited in previous studies, allowing the nature of the dynamics of hybridization between Atlantic eel species to remain inconclusive.

In this study, we revisit this question with the amplified fragment length polymorphism (AFLP) characterization of over 1100 eels representing different life stages from both continents, as well as multiple Icelandic locations. The AFLP technique can relatively easily reveal polymorphisms at hundreds of loci, and represents a very efficient technique for hybrid identification between closely related species (Congiu *et al.* 2001; Young *et al.* 2001; Bensch *et al.* 2002; Lucchini 2003; Bensch & Akesson 2005). Hybrid individuals were thus identified and classified in order to document the extent of hybridization, and to test for the occurrence of hybrids beyond the first generation. Second, we tested the null hypothesis of no differences in the

proportion of hybrid eels between the glass and yellow eel stages in Iceland. Finally, we documented the distribution of hybrids across both continents and within Iceland in order to test the null hypothesis of the random geographic distribution of hybrids.

Materials and methods

Samples

A total of 1127 eels were analysed. Icelandic samples ($n = 748$) were collected from 10 sites between 2000 and 2003, covering most of the known geographic distributional range in Iceland (Fig. 1, Table 1). From a previous study, genomic DNA of eels was available for eight sampling locations ($n = 379$); four from North America, three from continental Europe, and one from North Africa (Fig. 1, Table 1; see details in Wirth & Bernatchez 2001, 2003). These were considered baseline populations and used to identify the most informative AFLP loci, which were used for assessing the hybrid status of each individual eel.

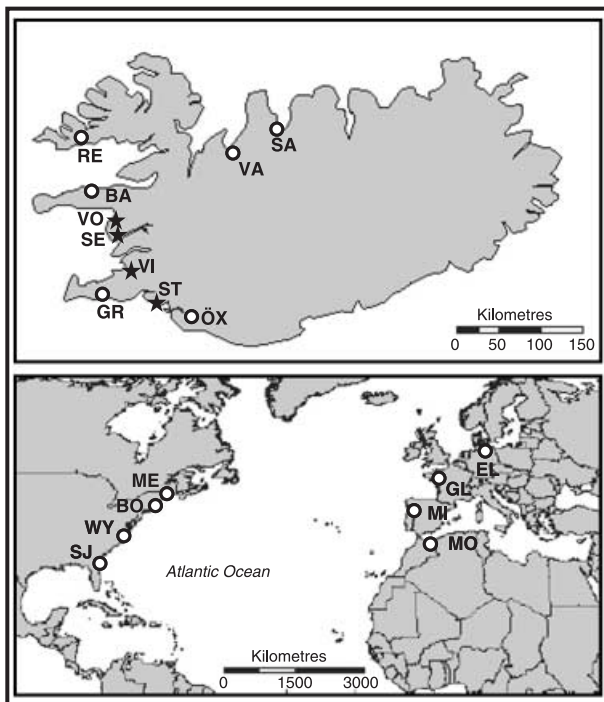


Fig. 1 Geographic distribution of eel sampling sites in Iceland (top panel), North America and Europe (bottom panel). Star symbols refer to locations where both glass eels and yellow eels were collected. Icelandic samples are Öxnalækur (ÖX); Stokkseyri (ST); Grafarvogur (GR); Vífilstadvatn (VI); Seljar (SE); Vogslækur (VO); Bár (BA); Reykhólar (RE); Vatnsdalur (VA); Sauðárkrókur (SA). North American eel locations: Medomak River (ME); Boston Harbor (BO); Wye River (WY); St-Johns River (SJ). European eel locations: Elbe River (EL); Grand Lieu Lake (GL); Minho River (MI); Moulouya oued (MO).

North American and continental European samples consisted of either all glass eels (BO, GL, MI, MO), or all yellow eels (ME, WY, SJ, EL). Yellow eels represent a mixture of different cohorts, and should therefore represent the average genetic composition at a given location over many years. Temporal genetic variation between cohorts has been reported (Dannewitz *et al.* 2005); however, it is very weak relative to interspecific differences (Wirth & Bernatchez 2003), and should therefore be inconsequential in assessing hybrid status. In Iceland, yellow eels were sampled at all 10 locations, whereas glass eels of several cohorts were available at four of these sites (ST, VI, SE, VO), which allowed the comparison of hybrid occurrence at two different life stages. Life stages, sampling years and sample sizes are detailed in Table 1.

AFLP genotyping

Fin clip and muscle tissue samples were digested and total genomic DNA extracted and purified using MultiScreen lysate clearing plate and MultiScreen₉₆ BAC plate from Montage BAC₉₆ Miniprep Kit (Millipore). The AFLP procedure of Vos *et al.* (1995) was followed in order to produce a DNA fingerprint for each of five selective primer combinations (*EcoRI*-AAC/*MseI*-CTA; *EcoRI*-AAC/*MseI*-CTT; *EcoRI*-AGG/*MseI*-CTT; *EcoRI*-ACT/*MseI*-CTC; *EcoRI*-ACT/*MseI*-CTT) with the *EcoRI* primer bearing the fluorescent dye). Each sample and primer combination was electrophoresed on a Base Station DNA Fragment Analyzer (MJ Research), with an internal lane GeneScan@-500 [ROX]TM size standard (Applied Biosystems). Tracking and peak identification was completed using CARTOGRAPHER DNA Fragment Analysis software (version 1.2.6). DNA fingerprints were smoothed and peaks with intensity over the threshold value were scored. Unambiguous loci were then identified and selected ($N = 373$) on the basis of reproducibility of fragment size (in base pairs), proximity to other loci, and signal intensity. The software AFLP-SURV version 1.0 (Vekemans 2002) was used to calculate the number and proportion of polymorphic loci, as well as the expected heterozygosity (H_E) assuming Hardy–Weinberg equilibrium within each sampling site and grouping (North America, Europe, Iceland). As the occurrence of hybrids may cause deviations from Hardy–Weinberg equilibrium assumption, expected heterozygosity should be interpreted cautiously. Difference in proportion of polymorphic loci between groups was tested by performing a nonparametric ANOVA (Kruskal–Wallis method) in SAS (release 8.02; SAS Institute).

Individual hybrid status

The power of selected loci to discriminate hybrid status was assessed using the population assignment simulator in AFLPOP (version 1.1; Duchesne & Bernatchez 2002). Based

Table 1 Life stage, sampling year, and sample size (n) for each sampling site separately. Proportion of polymorphic loci, expected heterozygosity (H_E), and absolute number of pure American (Am), F_1 , F_N , and pure European (Eu) for the American, European, and Icelandic samples as well as for each sampling site separately. G represents glass eels and Y, yellow eels. Dash signs (—) indicate no observations in the status category. Population abbreviations are defined in Fig. 1 legend

Population	Life stage	Year	n	Polymorphic loci (%)	H_E	Am	F_1	F_N	Eu
American									
ME	Y	99	45	54.7	0.193	43	—	2	—
BO	G	99	50	52.5	0.193	49	—	1	—
WY	Y	99	48	52.5	0.192	48	—	—	—
SJ	Y	99	50	49.1	0.187	50	—	—	—
Total			193	60.3	0.192	190	—	3	—
European									
EL	Y	99	49	52.8	0.193	—	—	—	49
GL	G	99	49	48.5	0.185	—	—	—	49
MI	G	99	45	54.4	0.189	—	—	—	45
MO	G	99	43	61.7	0.195	—	—	—	43
Total			186	61.1	0.191	—	—	—	186
Icelandic									
SA	Y	03	6	71.6	0.244	—	6	—	—
VA	Y	00	18	71.6	0.248	—	4	2	12
RE	Y	01	13	75.9	0.217	—	6	—	7
BA	Y	03	49	71	0.210	—	6	—	43
VO	G	00	50	53.6	0.196	—	9	3	38
VO	G	01	50	72.7	0.210	—	4	1	45
VO	G	02	49	52.3	0.191	—	1	—	48
VO	G	03	49	53.1	0.196	—	3	2	44
VO	Y	01	36	70	0.212	—	3	2	31
SE	G	01	48	71.8	0.211	—	7	2	39
SE	Y	01	46	69.4	0.209	—	14	4	28
VI	G	01	50	72.1	0.215	—	4	4	42
VI	Y	02	45	70.5	0.232	—	5	5	35
GR	Y	03	45	71.3	0.222	—	1	2	42
ST	G	01	46	68.6	0.207	—	1	2	43
ST	G	03	50	53.4	0.188	—	—	1	49
ST	Y	03	49	69.7	0.213	—	6	2	41
ÖX	Y	03	49	72.1	0.213	—	2	2	45
Total			748	68.1	0.214	—	82	34	632

on allelic frequencies observed in the North American and European baseline populations, the AFLPOP simulator randomly generated 1000 genotypes of each of the six following categories: pure American, pure European, first generation hybrids (F_1), backcrosses (BC_1 and BC_2), and second generation hybrids (F_2). Those 6000 simulated individuals were then blindly reassigned to their most probable category. Since the probability of erroneous assignment between the BC_1 , BC_2 and F_2 hybrid categories was relatively high (see Results), we subsequently combined these into a single category of later generation hybrids (F_N). Thus, we determined the most likely status of all available samples according to four categories: pure American, pure European, first generation hybrid (F_1), and later generation hybrids (F_N).

We then used a version of the STRUCTURE program adapted to dominant markers (version 2.2; D. Falush *et al.*, unpublished) to estimate individual's admixture propor-

tion, in this case the proportion of an individual's genome originating from either the American or the European eel gene pool. The Markov chain Monte Carlo (MCMC) algorithm implemented in the program clusters individuals into the presumed number of populations, minimizing the Hardy-Weinberg and gametic phase disequilibrium within populations, but also accounting for the genotypic ambiguity inherent in dominant markers such as AFLP (in which the presence of a band does not allow to distinguish between heterozygote and homozygote). Both American and European gene pools were assumed to represent two baseline populations ($k = 2$) without considering the prior information on the species of origin. American and European eel samples clustered very distinctively into two clusters, except for three American eels (see Results). This confirmed that the assumption of two populations in the admixture model could unambiguously discriminate

both species and allow the identification of individuals with hybrid ancestry. All American, European, and Icelandic samples were then run by STRUCTURE, assuming two populations for 50 000 iterations in the burn-in and 50 000 supplementary iterations.

Based on the 90% posterior probability interval of the admixture value, individuals were assigned to the four possible genetic status as follows: pure if their probability interval overlapped with 0 (pure American eel) or 1 (pure European eel), first generation hybrid (F_1) if their probability interval overlapped with 0.5 but not with either 0 or 1, and later generation hybrid if it did not overlap with either 0, 0.5 or 1. This methodology is conservative as it is most likely underestimating the number of later generation hybrids in favour of first generation hybrids and pure American or European eels. Theory predicts that, depending on the pedigree of individuals, some of the later generation hybrids can be characterized by a proportion of both parental gene pools (e.g. American and European eel) which is equal or close to that of a first generation hybrid or pure parental form (e.g. Epifanio & Philipp 1997).

STRUCTURE results were then compared to those obtained using the program NEWHYBRIDS (Anderson & Thompson 2002). Using Bayesian statistics and MCMC, this program computes the posterior probability that each individual belongs to one of the following classes: American, European, F_1 , BC_1 , BC_2 , and F_2 category. The clustering method of this latter program is based on an inheritance model defined in terms of genotype frequencies and is useful when populations are known to consist of pure and recent hybrids. Assuming Jeffrey's like priors for the mixing proportion and the allelic frequencies, the same data set was run for 50 000 iterations in the burn-in and 50 000 supplementary iterations.

Hybrid proportion vs. life stages and sampling sites

We determined whether there was a difference in the proportion of hybrids between the glass and yellow eel stages by performing a static cohort analysis on eels from the four Icelandic sites where both glass and yellow eels were sampled (ST, VI, SE, VO). The proportion of first generation hybrids, the proportion of later generation hybrids, and the total proportion of hybrids (combining the first and later generation) were compared between the glass and yellow eel stages (CATMOD procedure in SAS release 8.02; SAS Institute). Assuming a constant rate of hybridization, this cohort analysis is an efficient means to evaluate the relative hybrid fitness, allowing an estimate of the viability of hybrids in natural conditions (Bert & Arnold 1995). However, since we could not assess differential survival during the oceanic stages (larval stage prior to freshwater and silver eel stage following yellow eel metamorphosis), our interpretations are based on differential survival in

freshwater only. Moreover, there are currently no data available to evaluate the temporal variation in the proportion of hybrids reaching Iceland. In order to evaluate this possibility, we compared the proportion of hybrids between different time periods according to the life stages and the year eels were sampled. More specifically, we considered that yellow eel samples collected in 2000 were the oldest of all samples and all samples were classified into eight different time periods. We thus considered yellow eels collected in 2000 as belonging to the time period 1, yellow eels collected in 2001 to the time period 2, yellow eels collected in 2002 as eels from the time period 3, and yellow eels collected in 2003 belonging to the time period 4. We considered the glass eel samples collected in 2000 as eels from the time period 5, glass eels collected in 2001 as eels from the time period 6, glass eels collected in 2002 as eels from the time period 7, and finally glass eels collected in 2003 as eels from the time period 8. This allowed us to statistically test for heterogeneity in the occurrence of hybrids (F_1 , F_N , and both categories combined) between the different time periods. We used the chi-squared (χ^2) permutation method of Roff & Bentzen (1989) available from the executable MONTE in the REAP program (McElroy *et al.* 1992), with 1000 permutations to determine test significance.

Finally, heterogeneity in the geographic distribution of hybrids, both across continents and within Iceland, was first tested using a chi-squared (χ^2) permutation test on the occurrence of pure, first, and later generation hybrid eels. The association between the proportions of hybrids (F_1 and F_N categories combined) among Icelandic yellow eels and latitude was assessed by the Pearson correlation using SAS (release 8.02; SAS Institute). The two hybrid categories were pooled in order to reduce sampling bias due to the low occurrence of first or later generation hybrid in several sites after confirming the absence of significant difference in the geographic distribution between F_1 and F_N hybrids ($\chi^2 = 13.77$, $P = 0.12$). The correlation between mean length of eels in each site and latitude was assessed as well (Pearson correlation in SAS).

Results

Individual hybrid status

A total of 373 AFLP loci were resolved among all continental American and European eel samples, of which 120 had a frequency differential of 0.10 or higher between both species. A relatively high polymorphism was observed, whereby, 60% or more of the AFLP loci were polymorphic, depending on sample locations (Table 1). Namely, a highly significant difference in proportion of polymorphic loci was observed between the three sampling groups ($K = 10.14$, $P = 0.0063$), where Icelandic samples were characterized by a higher proportion of polymorphic loci compared

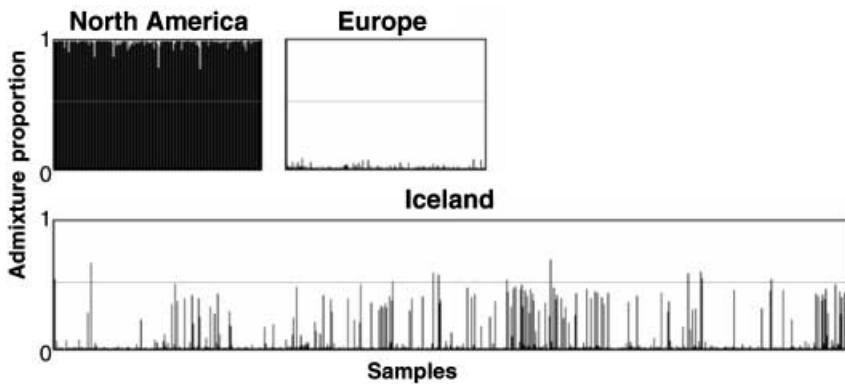


Fig. 2 Admixture proportions of North American, European, and Icelandic samples. Each individual is represented by a vertical bar; the proportion of black and white in each bar represents the proportion of the individual's genome from *Anguilla rostrata* (American) and *Anguilla anguilla* (European) ancestry, respectively. The dotted horizontal line indicates the 0.5 admixture level. Null values refer to admixture proportions smaller than 0.001, while 1 refers to admixture values larger than 0.999.

to North American and European samples, as would be expected for admixed vs. pure gene pools.

Based on the simulation and reassignment procedures performed with AFLPOP using the six eel categories (pure American, pure European, F_1 , F_2 , BC_1 , BC_2), the assignment success for pure American, pure European, and F_1 eels was 98.1%, 97.6%, and 83.2% respectively. However, misassignments in F_2 , BC_1 , and BC_2 were relatively high: 29.8%, 12.3%, and 12.6%, respectively. In order to reduce misassignment and also increase hybrid sample size for subsequent statistical analyses, F_2 and backcrosses were pooled into a single later generation hybrid category (F_N), which resulted in a misassignment rate of 8.1%, 7.6%, and 16.4% for the BC_1 , BC_2 , and F_2 , respectively, and a misassignment of 8.9% over all status categories.

The results of STRUCTURE revealed that the geographic distribution of admixture proportion differs substantially among continental American, European, and Icelandic samples (Figs 2 and 3, and Table 1). Samples from both continents were essentially composed of eels with either a pure American or European eel genome. Most Icelandic individuals were characterized by a genome of pure European ancestry. However, 15.5% of Icelandic samples were classified as admixed with variable degrees of their genome from American eel ancestry. Hybrid proportion within each yellow eel sample ranged from 6.6 to 100%. No pure American eel was observed among the Icelandic samples. Using this information to assess the genetic status of all individual eels based on the 90% posterior probability interval of the admixture value, the continental European samples consisted of only pure European eels, while the null hypothesis of pure American ancestry was rejected for three North American individuals. These had estimates of 7%, 20%, and 21% of their genome from European eel ancestry, and were identified as later generation hybrids (since their 90% probability interval did not overlap either with 0 or 0.5). In contrast, the null hypothesis of pure European eel ancestry was rejected for 15.5% ($n = 116$) of all Icelandic samples analysed. A total of 70.7% ($n = 82$) of putative hybrids in Iceland fell into the first generation

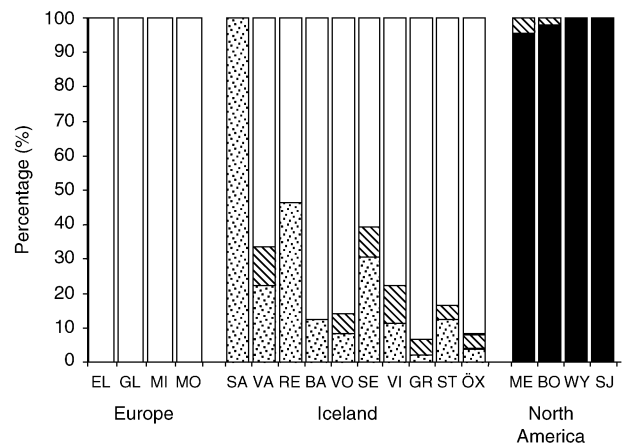


Fig. 3 Proportions of pure *Anguilla rostrata* (closed bars), pure *Anguilla anguilla* (open bars), first generation (dotted), and later generation hybrids (striped) within each sampling sites for continental Europe, Iceland, and North America. Population abbreviations are defined in Fig. 1 legend.

category, whereas 29.3% ($n = 34$) of all hybrids were classified as later generation hybrids, which confirmed the occurrence of hybrids beyond the first generation, and therefore, the viability and capacity of first generation hybrids to reproduce.

The individual status categories obtained from the software STRUCTURE were confirmed by the software NEWHYBRIDS, whereby 95% of the 1127 assignments were identical. Moreover, the proportion of each status categories detected by STRUCTURE and NEWHYBRIDS in each sampling location did not differ significantly and were highly correlated ($R^2 = 0.979$). Given such a high congruence between both methods, we retained and interpreted the detailed results of STRUCTURE.

Hybrid proportion vs. life stages and sampling sites

The static cohort analysis rejected the null hypothesis of no difference in the proportion of hybrids between glass and

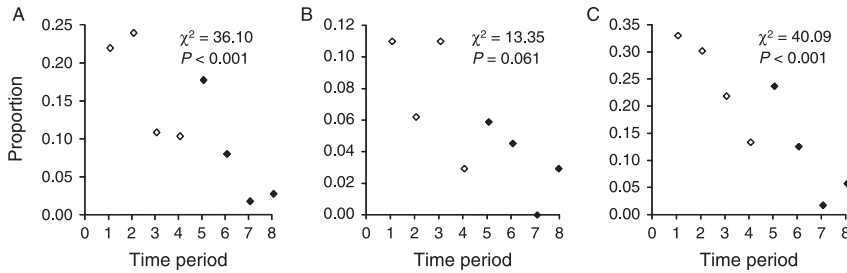


Fig. 4 Relationship between the proportion of F_1 (A), F_N (B), or F_1 and F_N combined (C) with time period (defined in Materials and methods section). Chi-square (χ^2) and P values are presented. Solid diamonds refer to yellow eel samples and open diamonds to the glass eel samples.

Table 2 Proportions of pure, first (F_1), and later generation hybrids (F_N) in the glass eel and yellow eel samples collected in Vogslækur (VO), Stokkseyri (ST), Vífilisstadavatn (VI), and Seljar (SE)

Site	Life stage	Pure European	Status category	
			F_1	F_N
VI	G	84.0	8.0	8.0
	Y	77.8	11.1	11.1
SE	G	81.3	14.6	4.2
	Y	60.9	30.4	8.7
VO	G	88.4	8.6	3.0
	Y	86.1	8.3	5.5
ST	G	95.8	1.0	3.1
	Y	83.7	12.2	4.1

yellow eels in Iceland. In seven out of eight comparisons, higher proportions of hybrids in yellow eel relative to glass eel samples were observed, the exception being the first generation hybrids in Vogslækur (VO) (Table 2). The categorical model analysis revealed no significant interaction between sampling site and life stage ($P = 0.5461$). Therefore, a main effect model, which did not consider the interaction between sampling sites and life stages, was run. The proportion of hybrids varied significantly among sampling sites ($P = 0.0036$) and between life stages ($P = 0.0155$), but sampling site had no significant effect on the difference in the proportion of hybrids observed between glass eels and yellow eels. On the other hand, the proportion of first generation hybrids and the proportion of first and later generation hybrids combined varied significantly between time periods ($P < 0.001$), while the variation in the proportion of later generation hybrids was near statistical significance ($P = 0.061$; Fig. 4). Moreover, a temporal trend was observed whereby the proportion of hybrids reaching Icelandic waters seemed to decrease from 2000 to 2003, based on the analysis of glass eel samples (time period 5–8). The addition of yellow eel samples (time period 1–4) further supported this pattern.

A χ^2 permutation test confirmed the nonrandom distribution of hybrids between Iceland and both continents ($\chi^2 = 59.16, P < 0.001$). A second analysis based only on Icelandic

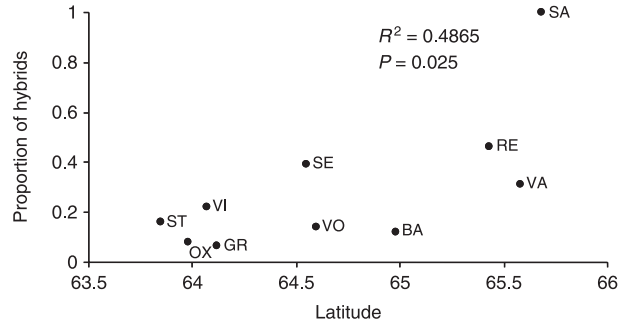


Fig. 5 Pearson correlation (R^2) between the proportion of hybrids and latitude (first and later generation hybrids combined) in Icelandic samples.

samples confirmed a highly significant nonrandom geographic distribution ($\chi^2 = 75.52, P < 0.001$). A significantly positive correlation was observed between the proportion of hybrids and latitude, explaining 48.7% of the variance in hybrid proportion ($P = 0.025$; Fig. 5). The proportion of explained variance remained high (39%) and near statistical significance ($P = 0.073$) even when removing the Sauárkrókur sample ($n = 6$) from analysis. The correlation between mean length of eels and latitude explained 21% of the variance and mean length tended to slightly increase with latitude, but was not significant ($P = 0.1826$).

Discussion

The objective of this study was to address three specific questions regarding the dynamics of hybridization between Atlantic eels. We first evaluated the extent of hybridization and tested for the occurrence of hybrids beyond the first generation. Second, we tested whether hybrids were randomly distributed across continents and among Icelandic sampling sites. Third, we tested for a difference in the proportion of hybrids between glass eel and yellow eel stages in Iceland. Our results provided evidence for (i) an overall hybrid proportion of 15.5% in Iceland, and the existence of hybrids beyond the first generation; (ii) a nonrandom geographic distribution of hybrids in the North Atlantic; and (iii) a higher proportion of first and later generation hybrids in the yellow eel stage compared

to the glass eel stage, as well as a significant latitudinal gradient in the proportion of hybrids in Icelandic freshwater. Below, we discuss the possible causes and consequences of these findings.

Extent of hybridization and existence of hybrids beyond F₁ generation

Avise *et al.* (1990) previously confirmed the existence of hybrid eels in Iceland. However, limited sample sizes precluded these authors from confidently estimating their proportion. Here, with the hundreds of markers and samples, we found that 15.5% of all Icelandic eels analysed were of hybrid origin. The broad coverage of sampling sites confirmed that hybrids are ubiquitous in Iceland, being present in all locations, representing nearly half of the eels analysed at some sampling sites (100% hybrids in the low sample size taken in SA). In contrast, we found no evidence for the occurrence of introgressed eels in Europe. This result was unexpected, given the previous report of a few European eels with intermediate vertebrae counts (Boëtius 1980). However, the abnormal vertebrae counts (≤ 109) were reported quasi-exclusively in northern Europe (Iceland, Denmark and Faroes, except for one report in both France and Spain). Our sampling coverage for the northern part of the species' distributional range in Europe precludes rigorously ruling out the occurrence of introgressed eels. On the other hand, our results suggest that a small proportion of American eels may be introgressed. Interestingly, the three eels for which a pure American origin was rejected were from the two northernmost sampling sites. This observation, although preliminary, corroborates previous observations that the probability of finding introgressed Atlantic eels may be higher at northern latitudes.

Based on previous studies, it has remained unknown whether hybrids are infertile, and therefore an evolutionary dead end or, in contrast, a possible avenue for maintaining gene flow between American and European eels. This study provides the first evidence for the relatively common occurrence of second or later generation hybrid eels, which represented approximately 30% of all hybrid specimens and approximately 5% of all Icelandic eels analysed. These proportions demonstrate that hybrids between American and European eels are viable, fertile, and can migrate back to the Sargasso Sea for reproducing beyond the first filial generation.

In situations of contentious species status, hybrid zones have long been recognized as a means to assess taxonomic status. Here, despite temporal and spatial reproductive sympatry and interbreeding, American and European eels remain reproductively isolated and almost entirely genetically distinct, therefore fulfilling the criteria of distinct biological species despite the potential for gene

flow between them (Coyne & Orr 2004). Since there are no obvious physical barriers during spawning to prevent hybridization between American and European eels, their persistence as distinct species could result from behavioural and/or ecological reproductive isolating barriers (e.g. Young *et al.* 2001; Taylor 2004), and/or as a result of sufficiently strong natural selection overcoming the homogenizing effects of gene flow (e.g. Schneider *et al.* 1999). In such a case, ecological speciation processes, rather than strict geographic isolation, could be responsible for maintaining the reproductive isolation between American and European eels. This, however, would not necessarily exclude a possible role for past allopatric separation in the maintenance of their reproductive isolation through endogenous processes, such as the accumulation of genotypic incompatibilities. The nucleotide sequence divergence estimate of 3.7% between the two monophyletic mitochondrial DNA clades that characterize American and European eels (Avise *et al.* 1986) suggests that they evolved in allopatry, perhaps during hundreds of thousands of generations, before their secondary contact. Indeed, the dual role of both historical contingency and ecological determinism has been previously reported in other fishes (Bernatchez *et al.* 1999; Taylor & McPhail 2000; Fraser & Bernatchez 2005).

Geographic distribution of hybrids

The second main observation of this study was the confirmation that both F₁ and later generation hybrid eels are found almost exclusively in Iceland. What factors may be responsible for this pattern? It has previously been documented that American and European eel larvae are not segregated during their migration (Kleckner & McCleave 1985 in Wang & Tzeng 2000) and consequently, it seems unlikely that Icelandic larvae would have their own migration route. Alternatively, interspecific differences in additive genetic basis for adaptive migratory behaviours and/or larval development could result in intermediate migration in hybrids (Rogers *et al.* 2002). Under this scenario, it would therefore be more likely that hybrids would end up in Iceland, which position is somewhat intermediate between both continents. The possibility for orientated swimming of the leptocephali larvae appears unlikely given the small size of the larvae, the high current velocity they must cope with, and the large distance to be covered (McCleave *et al.* 1998). Therefore, processes that could be responsible for the higher occurrence of hybrid eels in Iceland are more likely to be associated with developmental schedule and passive drift (McCleave 1987). Studies of daily growth increments indicated that there is a significant difference in larval duration between American and European eels: 200 and 350 days respectively (Wang & Tzeng 2000). Even if the daily deposition on the leptocephali otolith is still contentious (see Lecomte-Finiger 1994;

Cieri & McCleave 2000; Wang & Tzeng 2000), there is no doubt that the larval stage duration is longer for the European relative to the American eel. Given that fish larval development is partially under additive genetic control (Rogers & Bernatchez, in preparation), hybrids could spend intermediate time as a larva and therefore be more likely to successfully colonize freshwater habitats at intermediate locations between continents, such as Iceland. Differential growth rate between American (0.21 mm/day) and European eel larvae (0.15 mm/day) has also been reported (Wang & Tzeng 2000), which could further increase the probability of colonizing freshwater habitats in intermediate locations between continents. The premigration metamorphosis of yellow eels is timed at both the developmental and behavioural level (Haro 2003), such that eels must reach a minimum threshold size before undertaking their downstream migration (Oliveira 1999). Since minimal threshold size also apparently applies to leptocephali (Otake 2003), and given the interspecific difference in the average size of glass eel (Wang & Tzeng 2000), hybrids with intermediate growth may also be characterized by an intermediate time spent as larvae.

Hybrid proportion in Icelandic samples

Two distinct and confounding patterns were observed regarding the proportion and distribution of hybrids in Iceland. First, the proportion of hybrids was significantly higher in yellow eels compared to glass eels. Then, we also observed a variation in hybrid proportion with year of arrival in glass eels. Since yellow eels were collected approximately in the same time period as the glass eels, the higher hybrid proportion in the yellow eels could result from either a higher survival of eels in Icelandic freshwater and/or to a temporal trend in the reduction of hybrids reaching Iceland. Here, we discuss the potential scenarios that could be responsible for the patterns we observed, given the strengths and limitations of our data set.

Natural selection may influence the hybrids' relative fitness in terms of differential survival, and as such, modulate the observed proportion of hybrids. Examples where first generation hybrids (F_1) showed equal or higher fitness relative to at least one of the parental forms are common in plants (e.g. Burke *et al.* 1998; Orians *et al.* 1999; Campbell & Waser 2001; Campbell 2003; Song *et al.* 2004; Kirk *et al.* 2005), and several examples exist in animals as well (e.g. Grant & Grant 1992; Scribner 1993; Emms & Arnold 1997; Hotz *et al.* 1999; Parris 2001; Perry *et al.* 2001; Schweitzer *et al.* 2002). However, few studies have evaluated relative fitness beyond the first hybrid generation. Most studies directly comparing survival of first and later generation hybrids were performed in laboratory or controlled conditions (e.g. Moore & Koenig 1986; Saino & Villa 1992; Howard *et al.* 1993; Wang *et al.* 1997; Campbell *et al.*

1998; Vilà & D'Antonio 1998; Fritsche & Kaltz 2000; Good *et al.* 2000; Hauser *et al.* 2003; Burgess & Husband 2004; Gilk *et al.* 2004; Rosenfield *et al.* 2004; Facon *et al.* 2005). As a result, the evolutionary processes as well as the potential outcome of hybridization in natural settings remain largely unexplored in animals (Burke & Arnold 2001; but see Grant & Grant 1992; Emms & Arnold 1997; Perry *et al.* 2001; Schweitzer *et al.* 2002). Here, assuming that the proportion of hybrids arriving in Icelandic waters is relatively constant, the higher frequency of both F_1 and later generation hybrids in yellow eels relative to glass eels would indicate a higher relative survival of hybrids. In animals, higher hybrid survival has previously been documented for the Darwin's finches *Geospiza fortis*, *Geospiza scandens*, and *Geospiza fuliginosa*. Thus, Grant & Grant (1992) compared the survival of natural first and later generation hybrids to the parental forms and observed a higher survival in both categories. Other examples of natural hybrids with a higher relative fitness also exist (e.g. Moore & Koenig 1986; Saino & Villa 1992; Howard *et al.* 1993; Bert & Arnold 1995; Good *et al.* 2000; Rubidge & Taylor 2004). According to environment-dependent hybrid zone models, superior hybrid survival is more likely to be observed in novel habitats or in a novel combination of environmental factors (Howard *et al.* 1993). For instance, when surveying two ecologically differentiated taxa in sedge (*Carex curvula*), Choler *et al.* (2004) found that genotype integrity was maintained in optimal habitats, and that hybrids were favoured in marginal habitats. Genetic introgression was thus considered 'as a potential to widen a species' niche'. The situation may be similar for North Atlantic eels. Iceland, where hybrids are observed, is located at the limit of the northern distribution range of the European eel, and may therefore be considered as representing a marginal habitat relative to the typical environments to which pure European eels are mainly adapted to.

As recognized earlier by Grant & Grant (1996): 'the key to understand the spatial patterning of hybridization and the relative fitness of hybrids could lie in the ecology and breeding behaviour.' Here, we discuss hypothetical ecological and/or behavioural causes that could explain a higher hybrid survival in Iceland. Admittedly, however, the veracity of these hypotheses must await further experimental investigations. European freshwaters are warmer on average than Icelandic waters and the average length of the growing season is longer in continental Europe (Einarsson 1984). A higher hybrid survival raises the hypothesis that hybrid eels with hybrid genotypic combinations could be better adapted (either due to a shift in the mean or an increased variance of a phenotypic trait) than pure European eels to environmental conditions experienced in Iceland, thus increasing survival probability. For instance, American eels face colder winters on average relative to European eels (Seager *et al.* 2002). American eels are thus

potentially exposed to a larger range of temperatures than European eels, which could perhaps provide physiological advantages to hybrid eels relative to European eels in Iceland. Nonetheless, experiments on temperature physiological tolerance and adaptation of Atlantic eels in natural environments are lacking and difficult to realize. Eels in the laboratory typically stop feeding and gradually reduce their activity to complete torpor when exposed to low temperatures (Nyman 1972; Sadler 1979; Walsh *et al.* 1983; Linton *et al.* in press). Moreover, observation varies considerably from one laboratory experiment to another. For instance, Nyman (1972) reported that European eels did not feed or search for food when temperatures were lower than 14 °C and the same observation was made in the American eels (Barila & Stauffer 1980). In contrast, other studies mentioned lower feeding temperatures such as 8 °C (Walsh *et al.* 1983) or 10 °C (Bruun 1963 in Nyman 1972).

Second, a possible role for natural selection in explaining a higher proportion of hybrids in yellow eels relative to glass eels in Icelandic freshwaters was also suggested by a positive relationship between the proportion of hybrids and latitude. This observation raises the hypothesis of differential survival favouring hybrids that may correlate with a thermal latitudinal gradient. Thus, while there are no good records for freshwater temperature per se, it is known that mean water temperature correlates with mean air temperature, and that mean yearly air temperature decreases with latitude in our sampling area, resulting in shorter summers and colder winters at more northern latitudes (Einarsson 1984). Water temperature has a major influence on eels' metabolism (Sadler 1979), oxygen consumption, and enzyme activities (Walsh *et al.* 1983), as well as behaviours such as feeding, aggression, and habitat selectivity (Nyman 1972; Barila & Stauffer 1980). Therefore, different thermal optima between pure European and hybrid eels could result in differential performance and perhaps survival depending on the thermal regime. Although this possibility cannot be ruled out entirely, it seems less likely that the observed latitudinal gradient in the proportion of hybrids is associated with a variation in the proportion of hybrids arriving in Iceland. First, yellow eel samples were composed of eels from different cohorts, which should buffer the impact of the yearly variation. Second, we found no significant sampling site effect on the size of yellow eels that were collected. Although size only represents an approximation of age, the lack of association between mean eel size and latitude suggests that difference in age between yellow eels from different locations cannot explain alone the latitudinal pattern we observe.

Third, temporal changes in the relative survival of hybrids in the ocean associated with global warming could explain the apparent temporal decrease in the proportion of hybrids arriving in Icelandic waters. For instance, Perry *et al.* (2005) demonstrated that many exploited and non-

exploited fishes from the North Sea, have responded markedly to recent increases in sea temperature, whereby the distribution of 21 out of 36 species surveyed shifted either in mean latitude, depth or both over the last 25 years. In Atlantic eels, the Den Oever glass eel recruitment index declined markedly since the late 1980s, which coincided with consistently positive sea surface temperature anomalies since then (ICES 2001; Knights 2003). Under the hypothesis that the thermal physiological optima is lower for hybrids relative to pure eel larvae, higher temperature could result in increased relative survival of pure European eels, and in an apparent reduction of the proportion of hybrid eels arriving in Icelandic waters.

An additional, nonexclusive cause of the variation in the proportion of hybrids arriving in Iceland could reside in the modification of the oceanic circulation (Knights 2003; Wirth & Bernatchez 2003). Thus, the ongoing warming of the Sargasso Sea/Sub-Tropical Gyre is hypothesized to inhibit spring thermocline mixing and nutrient mixing, which would have marked impact on productivity in the spawning area of the Atlantic eels and could reduce the amount of food available for the leptocephali (Bates 2001). Moreover, modifications in oceanic currents could reduce transport rate and hence probably prolong migration of leptocephali, exacerbating impacts of low nutrition and exposing them for a longer period to predation (Knights 2003). Under the hypothesis that hybrids are genetically intermediate between European and American eels, and knowing that European eels are adapted to a longer oceanic migration period relative to American eels (Arai *et al.* 2000; Wang & Tzeng 2000), it is plausible that ongoing changes in oceanic circulation could result in a lower proportion of hybrids reaching Icelandic waters relative to pure European eels.

Besides differential survival between hybrids and pure European eels, both in freshwater and in the ocean, additional factors may explain the higher proportion of hybrids we observed in yellow eel compared to glass eel samples. Spatial variation in hybrid zone dynamics has been reported in several species (e.g. Bleeker & Hurka 2001; Williams *et al.* 2001; Watano *et al.* 2004; Aldridge 2005). In the same way, temporal variation in the extent of spatial overlap could also result in variation in the extent of hybridization. For instance, Watano *et al.* (2004) found an important role of the size and position of the distribution gap in two *Pinus* species to explain the differences in level and pattern of introgression in two distinct contact sites. Moreover, Emelianov *et al.* (2001) proposed that year-to-year variation in population densities could be an important factor leading to temporal variation in natural hybridization. Clearly, recruitment in American and European eels has declined sharply in recent decades (Castonguay *et al.* 1994; Dekker 2000; Haro *et al.* 2000; ICES 2001), possibly by up to 90% in some European rivers (Dekker 2003). We propose that

such demographic decline could decrease densities of eels on spawning grounds in the Sargasso Sea. Decreased densities could in turn reduce the area of overlap during spawning and therefore the number of hybrids being produced. The decreasing trend in hybrid proportion arriving in Icelandic waters from 2000 to 2003 tends to support this hypothesis, although a longer time series will be necessary to verify it.

Admittedly, this study does not allow to confidently conclude on the nature of possible causes for the higher proportion of hybrids observed in the yellow eels compared to glass eels in Iceland. However, since pre- and postmating isolating barriers are nonexclusive processes, we propose that the combined effect of both differential survival of hybrids and variation in hybridization rate through time may best explain the patterns we observe. Indeed, climate change is impacting many environmental features and could have a determinant effect on the outcome of natural hybridization in Atlantic eels. Moreover, the complexity of Atlantic eels' life cycle, which comprises several critical stages, may exacerbate the impact of environmental conditions on the production and survival of hybrids. Clearly, experimental investigations, as well as additional field data, will be necessary for elucidating the role of both selective and demographic factors influencing the dynamics of natural hybridization in Atlantic eels.

Implications for natural hybridization studies

The diverse array of outcomes in hybridizing species calls for more studies of introgressive hybridization in natural environments. This study added to the diversity of possible outcomes of hybridization in that hybridization between American and European eels results in a very unique case of hybrid zone, whereby hybrids are located thousands of kilometres away from where interbreeding occurs. To our knowledge, it is the first documentation of such a hybrid zone. Second, this study illustrated that interspecific hybridization is a very dynamic process, whereby the combination of both pre- and postmating mechanisms may result in pronounced spatio-temporal fluctuations in the proportion of hybrids found in nature. It also suggests that post-F₁ hybrids may have higher fitness than parental forms under specific circumstances, and demonstrates that introgressive hybridization is a possible avenue for maintaining gene exchanges between American and European eels. Overall, our results add to the increasing number of studies showing that natural hybridization may play a significant role in adaptation and evolution.

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This research is part of Vicky Albert's MSc thesis, which aimed at studying the dynamics of natural hybridization in Atlantic eels. Bjarni Jonsson's interests are in the field of evolutionary and molecular ecology, speciation, effects of climatic change and life history adaptation. Louis Bernatchez supervised Vicky Albert's MSc thesis. LB's interests relate to the understanding of patterns and processes of molecular and organismal evolution, and their relevance to conservation.
