JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12750

Low reproductive isolation and highly variable levels of gene flow reveal limited progress towards speciation between European river and brook lampreys

Q. ROUGEMONT*†[1], A. GAIGHER*†‡[1], E. LASNE§¶, J. CÔTE*†, M. COKE**, A.-L. BESNARD*†, S. LAUNEY*† & G. EVANNO*†

*UMR 985 Ecologie et Santé des Ecosystèmes, INRA, Rennes, France
†UMR ESE, Agrocampus Ouest, Rennes, France
‡Laboratory for Conservation Biology, Department of Ecology and Evolution, University of Lausanne, Biophore, Switzerland
§Muséum National d'Histoire Naturelle, CRESCO, Dinard, France
¶UMR CARRTEL, INRA, Thonon-les-Bains, France
**Unité Expérimentale d'Ecologie et d'Ecotoxicologie Aquatique, INRA, Rennes, France

Keywords:

genetic structure;
Lampetra;
life-history strategy;
parapatry;
reproductive barrier;
sympatry.

Abstract

Ecologically based divergent selection is a factor that could drive reproductive isolation even in the presence of gene flow. Population pairs arrayed along a continuum of divergence provide a good opportunity to address this issue. Here, we used a combination of mating trials, experimental crosses and population genetic analyses to investigate the evolution of reproductive isolation between two closely related species of lampreys with distinct life histories. We used microsatellite markers to genotype over 1000 individuals of the migratory parasitic river lamprey (*Lampetra fluviatilis*) and freshwater-resident nonparasitic brook lamprey (*Lampetra planeri*) distributed in 10 sympatric and parapatric population pairs in France. Mating trials, parentage analyses and artificial fertilizations demonstrated a low level of reproductive isolation between species even though size-assortative mating may contribute to isolation. Most parapatric population pairs were strongly differentiated due to the joint effects of geographic distance and barriers to migration. In contrast, we found variable levels of gene flow between sympatric populations ranging from panmixia to moderate differentiation, which indicates a gradient of divergence with some population pairs that may correspond to alternative morphs or ecotypes of a single species and others that remain partially isolated. Ecologically based divergent selection may explain these variable levels of divergence among sympatric population pairs, but incomplete genome swamping following secondary contact could have also played a role. Overall, this study illustrates how highly differentiated phenotypes can be maintained despite high levels of gene flow that limit the progress towards speciation.

Introduction

Understanding the process of speciation, that is the evolution of reproductive isolation, is a central issue in evolutionary biology. Reproductive barriers among populations can be due to genetic incompatibilities that cause intrinsic reproductive isolation and/or divergent selection that produces extrinsic reproductive isolation (Coyne & Orr, 2004; Seehausen *et al.*, 2014). In allopatry, the cumulative effects of selection (including sexual selection), genetic drift and mutation can lead to speciation. Alternatively, sympatric speciation has been regarded as less likely as gene flow between nascent species will contribute, *via* recombination, to continuously break down associations between alleles linked to

Correspondence: Guillaume Evanno, UMR 985 Ecologie et Santé des Ecosystèmes, INRA, 35042 Rennes, France.
Tel.: +33 2 23 48 54 45; fax: +33 2 23 48 54 40;
e-mail: guillaume.evanno@rennes.inra.fr
[1]Both authors contributed equally to this work.

divergent adaptation (Felsenstein, 1981; Kirkpatrick & Ravigné, 2002). In parapatry, reproductive isolation can be maintained only under restricted values of gene flow (Bank *et al.*, 2012). The spatial context of speciation could thus greatly influence the evolution of reproductive isolation by constraining or facilitating gene flow (Sobel *et al.*, 2010; Marie Curie Speciation Network *et al.*, 2012).

Studies under these different geographic settings (sympatry, allopatry and parapatry) have provided evidence for the role of natural selection in promoting speciation (Jiggins *et al.*, 2001; Nosil *et al.*, 2002; McKinnon *et al.*, 2004; Barluenga *et al.*, 2006; Langerhans *et al.*, 2007; Soria-Carrasco *et al.*, 2014) and have shown variable levels of divergence from panmixia to complete reproductive isolation resulting in a divergence continuum (Hendry, 2009; Nosil *et al.*, 2009a; Præbel *et al.*, 2013). It remains challenging to discriminate the respective roles of gene flow, mutation and population size relative to the action of natural selection along this continuum (Barrett & Hoekstra, 2011). The relative importance of these factors is usually assessed by studying replicate pairs of taxa or populations either in sympatry (Nosil *et al.*, 2009b; Gagnaire *et al.*, 2013; Powell *et al.*, 2013) or in parapatry (Berner *et al.*, 2009; Kaeuffer *et al.*, 2012; Roesti *et al.*, 2012) but rarely in both situations simultaneously. In most cases, results have been interpreted as evidences of recent and independent parallel divergence due to ongoing (ecological) selection and the role of demographic history has been usually overlooked (Bierne *et al.*, 2013). However, it can be particularly difficult to disentangle the role of different past demographic events that can leave similar signatures in the genetic makeup of present-day populations (e.g. Hewitt, 1996, 2011). For instance, it is often challenging to distinguish between primary divergence in sympatry vs. a secondary contact following differentiation in allopatry because neutral markers often used for demographic inference may converge to the same equilibrium under both scenarios (Endler, 1977; Barton & Hewitt, 1985; Bierne *et al.*, 2013). In addition, population divergence after primary or secondary contact does not always lead to complete reproductive isolation (sensu Mayr, 1947) and to the formation of a new species (Mallet, 2008; Hendry, 2009; Elias *et al.*, 2012; Nosil, 2012). As a result, it is important to combine experimental approaches analysing reproductive barriers with inferences of gene flow in replicated population pairs to measure the level of reproductive isolation between putative species (e.g. Dey *et al.*, 2012; Sobel & Streisfeld, 2015). Here, we used such an integrative approach by focusing on both parapatric and sympatric population pairs in an emerging model species that displays two extremely different life-history strategies.

Lampreys are ancient jawless vertebrates (agnathans) in which most genera are described as 'paired' species (Zanandrea, 1959) with divergent life histories, which represent putative cases of ecological speciation (Salewski, 2003). These paired species reproduce in freshwater but have extremely different feeding strategies at the adult stage: some taxa are parasitic (haematophagous) and anadromous (i.e. migrate at sea), whereas others are nonparasitic and freshwater-resident. Larvae from both taxa are morphologically undistinguishable, but adults can be distinguished mainly by the larger body size of parasitic taxa (Hardisty & Potter, 1971; Vladykov & Kott, 1979; Potter, 1980). Paired species are phylogenetically closely related, and it is usually assumed that nonparasitic species derived from their parasitic counterparts (Zanandrea, 1959; Vladykov & Kott, 1979; Docker, 2009).

In Western Europe, the nonparasitic brook lamprey (*Lampetra planeri*, Bloch, 1784) and the parasitic river lamprey (*Lampetra fluviatilis*, Linnaeus, 1758) display a low-to-moderate genetic differentiation as measured with allozymes (Schreiber & Engelhorn, 1998), mitochondrial DNA and microsatellite markers (Espanhol *et al.*, 2007; Blank *et al.*, 2008; Mateus *et al.*, 2011; Bracken *et al.*, 2015). In addition, the high viability of F1 hybrid larvae (Hume *et al.*, 2013a), communal spawning of both species on the same nest (Huggins & Thompson, 1970; Lasne *et al.*, 2010) and sneaking behaviour of males towards spawning females from the other species have been observed (Hume *et al.*, 2013b). These results led to the hypothesis that brook and river lampreys may represent alternative life-history strategies (or ecotypes) within a single species (Beamish, 1987; Yamazaki *et al.*, 2006; April *et al.*, 2011; Docker *et al.*, 2012; Knebelsberger *et al.*, 2015). Alternatively, it was argued that the divergence between the two species may be very recent (Docker *et al.*, 1999; Salewski, 2003; Espanhol *et al.*, 2007; Okada *et al.*, 2010). However, Mateus *et al.* (2013) found a strong genome-wide divergence between *L. fluviatilis* and *L. planeri* sampled in a single river and concluded on the taxonomic validity of the two species. In addition, the different size of adults of both species has been hypothesized to induce size-assortative mating leading to reproductive isolation (Beamish & Neville, 1992). Nevertheless, this hypothesis has never been thoroughly investigated by testing whether the sneaking behaviour of *L. planeri* males can lead to the fertilization of *L. fluviatilis* eggs and the production of viable hybrids (Hume *et al.*, 2013b). The various population genetic studies led so far have also rarely distinguished the situations of sympatry and parapatry, and it remains unclear whether the moderate-to-strong levels of genetic differentiation observed between both species within the same river were due to reproductive isolation in sympatry, isolation by

distance (IBD) or anthropogenic barriers (e.g. dams or weirs) in parapatry (Schreiber & Engelhorn, 1998; Mateus *et al.*, 2013; Bracken *et al.*, 2015).

In this study, we used an integrative approach combining experimental measures of reproductive isolation and estimates of gene flow in replicated population pairs to better understand the evolution of divergence between *L. fluviatilis* and *L. planeri*. Hereafter we use the term 'species' as it is the current taxonomic status of these lampreys, but we acknowledge that other terms such as 'ecotypes' or 'forms' may also be appropriate. First, we measured the reproductive success of *L. fluviatilis* and *L. planeri* males under semi-natural conditions where only *L. fluviatilis* females were present to test whether size-assortative mating induces a strong prezygotic isolation. Second, we realized *in vitro* fertilizations of *L. fluviatilis* eggs with semen from *L. fluviatilis* and *L. planeri* males to compare the fertilization and hatching rates of eggs from intra- and interspecific crosses. Third, we performed a large-scale population genetic analysis including five sympatric and five parapatric population pairs to infer the level of gene flow between species and among populations within species. We hypothesized that if the level of reproductive isolation between *L. fluviatilis* and *L. planeri* is high, a strong level of pre- and post-zygotic isolation will be observed in our experiments as well as a low level of gene flow among sympatric populations. Alternatively, if *L. fluviatilis* and *L. planeri* were ecotypes of a single species at a very early stage of divergence or lineages subject to a secondary contact after a period of allopatric divergence, we expected a low reproductive isolation combined with high levels of gene flow in natural populations.

Materials and methods

Reproductive isolation: reproductive success under semi-natural conditions

We quantified the reproductive success of *L. fluviatilis* and *L. planeri* males under semi-natural conditions where only *L. fluviatilis* females were present. We aimed at testing whether size-assortative mating may prevent any mating between *L. planeri* males and *L. fluviatilis* females. Four *L. planeri* males, two *L. fluviatilis* males and two *L. fluviatilis* females were caught by electrofishing in March 2013 on the downstream part of the Oir River (Fig. 1). Individuals were kept in a 300-L tank with 3–5 cm of fine gravel (0.5–1.5 cm diameter) in a recirculated water system. Temperature was set at 12 ± 1 °C with a 12 : 12 photoperiod. After spawning of both females, 129 larvae as well as tissue samples from each adult were collected and genotyped using 13 microsatellite markers (Gaigher *et al.*, 2013). Parentage analyses were performed with CERVUS 3.0 (Kalinowski *et al.*, 2007) using the trio logarithm of the odd score, a 95% confidence level and allowing either no or up to two mismatches between putative parents and offspring.

Reproductive isolation: artificial fertilization

We performed *in vitro* fertilizations of *L. fluviatilis* eggs with sperm from both species. Six *L. fluviatilis* females were crossed with four males of each species in a full-factorial design producing 48 sib groups. Eight males and three females were captured by electrofishing in the Oir River (Fig. 1), whereas three other females were collected in the Loire River by a

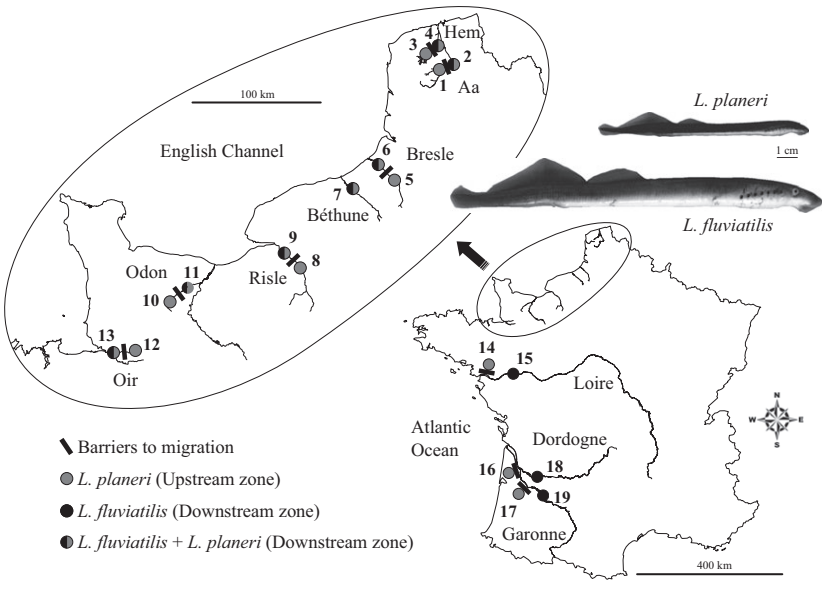

Fig. 1 Map showing the sampling sites in France (numbers match those given in Table 1).

Table 1 Genetic diversity estimates of *Lampetra fluviatilis* (*Lf*, $n = 523$) and *Lampetra planeri* (*Lp*, $n = 500$) populations based on 13 microsatellite loci for each site and year.

	Site	*N*	Mean size (mm)	Ar	An	Ho	He	Fis
Populations								
Lf Aa D 2014	2 (S)	34	310.5	3.511	3.846	0.504	0.514	0.019
Lf Hem D 2014	4 (S)	30	300.1	3.313	3.769	0.497	0.504	0.013
Lf Bre D 2010	6 (S)	38	335.9	3.415	3.692	0.467	0.480	−0.005
Lf Bre D 2011	6 (S)	41	334.6	3.252	3.615	0.478	0.484	−0.004
Lf Bet D 2014	7 (S)	17	306.8	3.761	3.538	0.475	0.514	0.078
Lf Ris D 2010	9	19	NA	3.555	3.769	0.526	0.517	−0.018
Lf Ris D 2011	9	40	320.4	3.212	3.615	0.478	0.500	0.041
Lf Ris D 2014	9	35	315.9	3.769	3.769	0.515	0.503	0.024
Lf Odo D 2011	11	32	316	3.207	3.692	0.502	0.486	−0.035
Lf Oir D 2010	13 (S)	34	222	3.399	3.846	0.554	0.542	−0.048
Lf Oir D 2011	13 (S)	40	229.4	3.188	3.692	0.506	0.505	−0.009
Lf Oir D 2014	13 (S)	30	222.9	3.462	3.462	0.505	0.52	−0.03
Lf Loi D 2010	15	32	290.8	3.089	3.308	0.468	0.461	−0.016
Lf Loi D 2011	15	32	NA	3.223	3.38	0.483	0.507	0.047
Lf Dor D 2010	18	39	250.7	3.068	3.308	0.527	0.486	−0.110*
Lf Dor D 2011	18	15	291.9	3.138	3.154	0.543	0.500	−0.09
Lf Gar D 2011	19	15	258.3	3.210	3.231	0.547	0.542	−0.016
Lp Aa D 2014	2 (S)	30	129.9	2.921	3.231	0.563	0.528	−0.068
Lp Aa U 2014	1	39	129	3.091	3.462	0.492	0.522	0.059
Lp Hem D 2014	4 (S)	39	155	3.738	3.462	0.469	0.477	0.017
Lp Hem U 2014	3	26	126	2.996	2.923	0.504	0.471	−0.071
Lp Bre D 2011	6 (S)	21	132.4	2.763	2.923	0.349	0.347	−0.005
Lp Bre U 2011	5	28	133.1	2.690	2.923	0.342	0.345	0.009
Lp Bet D 2014	7 (S)	17	144	3.791	3.385	0.482	0.472	−0.023
Lp Ris D 2011	9 (S)	1	147	NA	NA	NA	NA	NA
Lp Ris U 2011	8	16	143.3	3.323	3.385	0.335	0.335	−0.114
Lp Ris U 2014	8	28	136.5	3.770	3.769	0.466	0.435	0.066
Lp Odo D 2011	11	2	117.5	NA	NA	NA	NA	NA
Lp Odo U 2011	10	33	124.7	2.257	2.385	0.343	0.329	−0.043
Lp Oir D 2010	13 (S)	34	112	3.114	3.385	0.503	0.511	−0.043
Lp Oir D 2011	13 (S)	17	125.2	2.980	3.077	0.516	0.481	−0.075
Lp Oir D 2014	13 (S)	23	129.1	3.154	3.154	0.492	0.534	−0.087
Lp Oir U 2011	12	35	114.6	2.929	3.154	0.481	0.495	0.027
Lp Oir U 2014	12	31	122.8	3.000	3.000	0.458	0.466	−0.018
Lp Cen U 2011	14	33	163.3	2.248	2.462	0.257	0.262	0.008
Lp Jal U 2011	16	17	117.9	2.699	2.769	0.443	0.457	0.031
Lp Sau U 2011	17	30	109	2.459	2.538	0.405	0.400	−0.012
Species (mean)								
L. fluviatilis			287.9	3.270	3.570	0.502	0.501	−0.002
L. planeri			131.2	2.871	3.077	0.446	0.444	−0.005

Site numbers refer to Fig. 1, *N*, number of individuals; Ar, allelic richness (based on resampling of 14 individuals); An, number of alleles (averaged over loci); Ho, observed heterozygosity; He, expected heterozygosity; Fis, inbreeding coefficient (*significant deviation from the Hardy–Weinberg equilibrium); (S), U, and D refer to sympatric, upstream and downstream sites, respectively.

Bet, Béthune; Bre, Bresle; Ris, Risle; Odo, Odon; Oir, Oir; Loi, Loire; Cen, Cens; Dor, Dordogne; Gar, Garonne; Jal, Jalle de Tiquetorte; Sau, Saucats. Jalle de Tiquetorte is a tributary of the Gironde estuary that is common to Garonne and Dordogne rivers. The Bresle, Risle and Jalle de Tiquetorte *L. planeri* samples from 2011 include 1, 2 and 5 juvenile individuals, respectively. The *L. planeri* Risle U, Hem U and Aa U samples from 2014 are composed only of juveniles. The Odon *L. fluviatilis* samples include seven juveniles. All other samples include adult individuals only.

professional fisherman. We used females from two genetically differentiated populations (Oir and Loire) to discriminate the effects of outbreeding among populations and reproductive isolation between species (Waser & Price, 1985, 1994; Schierup & Christiansen, 1996). We used an experimental design similar to that of Rodríguez-Muñoz & Tregenza (2009) presented in detail as supporting information. The fertilization success for each sib group was estimated 3 h after fertilization based on the presence of a perivitelline space in the eggs (Ciereszko *et al.*, 2000). We then measured the hatching rate at the individual level in microplates

using only successfully fertilized eggs to avoid confounding dead and nonfertilized eggs.

Generalized linear mixed models with a binomial error family were used to test the influence of the cross type (within species ♀*Lf* × ♂*Lf* vs. between species ♀*Lf* × ♂*Lp*) and maternal population (Oir vs. Loire) on fertilization success and hatching rate. Cross type, population and cross type × population were considered as fixed effects. Sire, dam, sire × dam and microplates (only for hatching rate) were treated as random effects. To test the significance of each factor on the response variable, we compared models including or not the focal variable using likelihood ratio tests (LRTs) based on a chi-square distribution (Zuur *et al.*, 2009). Differences among populations were also investigated for each cross type separately to account for significant cross type × population interactions. Statistical analyses were performed with the lme4 package (Bates *et al.*, 2014) in the R software (R Development Core Team 2011). Experiments were approved by the Ethics Committee for Animal Experiment of Rennes (file number: R-2012-EG-02).

Sampling for population genetic analyses

A total of 1023 lampreys were sampled in 19 sites spread over 13 rivers in France during the spawning period in 2010, 2011 and 2014 (Table 1, Fig. 1). Individuals were anesthetized with benzocaine and measured to the nearest millimetre, and a fin clip was collected on each specimen and preserved in 95% EtOH. We sampled both species in sympatry simultaneously on the same spawning ground in the Aa, Béthune and Oir rivers. Two other sites were also considered as sympatric: the Bresle River, where the two species were captured 8 km apart (with no anthropogenic barrier in between), and the Hem River, where *L. planeri* individuals were sampled above a dam occasionally passable for *L. fluviatilis*, depending on water level. We also sampled *L. planeri* in five parapatric upstream sites inaccessible to *L. fluviatilis* due to dams or weirs: Risle, Odon, Cens, Saucats and Jalle de Tiquetorte rivers. Such upstream sites inaccessible to *L. fluviatilis* were also sampled in the Aa, Hem, Bresle and Oir rivers (i.e. the sympatric sites) to quantify the within-river genetic variability of *L. planeri*.

Molecular and statistical analyses

Genomic DNA was extracted from fin clips using a modified Chelex protocol (Estoup *et al.*, 1996). Genotyping was performed with 13 microsatellite markers specifically developed for *L. planeri* and *L. fluviatilis* (Gaigher *et al.*, 2013). Allelic richness (Ar), observed heterozygosity (Ho), expected heterozygosity (He) and inbreeding coefficient (Fis) for each population were calculated using FSTAT 2.9.3 (Goudet, 2001). The number of private alleles (Pa) was estimated with GENALEX 6.5 (Peakall & Smouse, 2012). Exact tests implemented in GENEPOP 4.1.0 (Rousset, 2008) were used to test the Hardy–Weinberg equilibrium (HWE) and the linkage disequilibrium. The Bonferroni correction was used to adjust the significance level for multiple tests (Rice, 1989; $\alpha = 0.05$). Potential differences of expected heterozygosity (He) and allelic richness (Ar) between species were investigated using the permutation test implemented in FSTAT (15 000 permutations). Differences of He and Ar between *L. fluviatilis* and *L. planeri* from the same river and the same year were further tested with Wilcoxon paired signed-rank tests (using values per locus) in R. F_{ST} among populations within species was estimated by θ (Weir & Cockerham, 1984), and pairwise values were tested using 17 000 permutations and the Bonferroni correction in FSTAT. An analysis of molecular variance (AMOVA) was performed with ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010) to quantify the hierarchical distribution of genetic variability between the two species, among populations within each species and within populations. The significance of variance components was tested with 15 000 permutations.

Genetic structure and gene flow

The genetic structure was analysed with the Bayesian clustering approach implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). The number of genetic clusters (k) varied from 1 to 12, and 20 independent replicates per k value were performed. Markov chain Monte Carlo simulations were based on 250 000 burn-in followed by 500 000 iterations using an admixture model with correlated allele frequencies (Falush *et al.*, 2003). The most likely number of clusters was determined with the estimated log likelihood Ln Pr($X|K$) and the ΔK method (Evanno *et al.*, 2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Plots were drawn using DISTRUCT 1.1 (Rosenberg, 2004) by averaging individual membership values over the 20 runs for the best k. A global analysis using the full data set of 1023 individuals was performed, and then, the level of divergence was investigated within population pairs and in each species separately.

Ongoing gene flow between sympatric and parapatric pairs of *L. planeri* and *L. fluviatilis* was estimated using BAYESASS 1.3 (Wilson & Rannala, 2003). Delta values for migration rates, inbreeding coefficient and allele frequencies were optimized to obtain acceptance rates between 40% and 60% of the total number of iterations to ensure proper chain mixing. The program was run with a burn-in of 2 000 000 followed by 7 000 000 iterations with five runs initiated with random seed numbers.

To test for a pattern of IBD, the correlation between pairwise geographic and genetic distances

measured by $F_{ST}/(1 - F_{ST})$ (Rousset, 1997) was tested using a Mantel test in R with 10 000 permutations. Geographic distances between each sampling site along coastline and within rivers were computed using ArcGIS 9.3.

Results

Reproductive success under semi-natural conditions

The 129 larvae were all successfully assigned with a 95% confidence level and up to two locus mismatches to a pair of parents, and 73 of 129 were assigned with no locus mismatches between parents and offspring (Fig. 2). From these 73 larvae, 59 (81%) were assigned as pure *L. fluviatilis* and 14 as hybrids (19%). Each *L. planeri* male produced two to four offspring, whereas the two *L. fluviatilis* males sired 35 and 24 offspring, respectively. The two *L. fluviatilis* females produced 47 and 26 offspring, respectively.

Artificial fertilization: fertilization success and hatching rates

Fertilization rates (± SE) of eggs from both homospecific (♀*Lf* × ♂*Lf*) and heterospecific (♀*Lf* × ♂*Lp*) crosses were extremely high: 95.5 ± 3.2% and 95.8 ± 3.8%, respectively. We found a significant interaction between cross type and maternal population on fertilization success (Table S1). However, considering each cross type separately, no population effect was found for both hetero- and homospecific crosses (LRT $\chi^2 = 3.28$, $P = 0.07$; LRT $\chi^2 = 1.50$, $P = 0.22$). Hatching rates were also extremely high: all eggs from homospecific crosses successfully hatched (100%) and only 5 of 563 eggs (99.1 ± 1.74%) from heterospecific crosses failed to hatch. Given the lack of variance in homospecific crosses, we could not statistically test for an effect of cross type on hatching rate. Considering heterospecific crosses, no population effect was found (LRT $\chi^2 = 0$, $P = 1$).

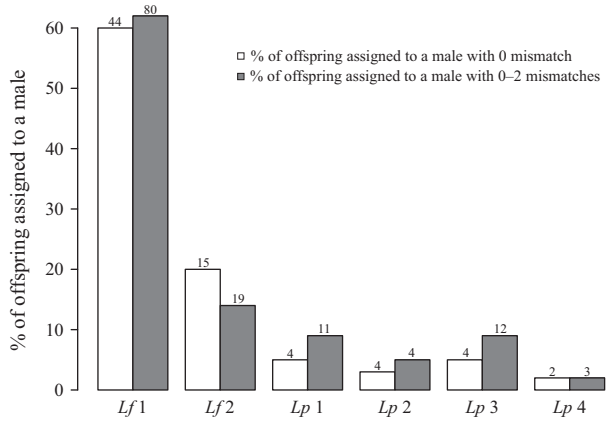

Fig. 2 Reproductive success of four *Lampetra planeri* and two *Lampetra fluviatilis* males under semi-natural conditions after spawning with two *L. fluviatilis* females. White bars represent the percentage of offspring assigned to a male at the 95% confidence level when no mismatch was allowed in assignment tests (73 individuals assigned). Grey bars represent the percentage of offspring assigned to a male at the 95% confidence level when up to two mismatches were allowed (129 individuals assigned). Numbers on top of each bar depict the absolute number of offspring assigned.

Within-species and population genetic diversity

A total of 1023 individuals were successfully genotyped at 13 loci. The number of alleles per locus varied from 4 to 12, for an average of 5.8 (Table S2). No linkage disequilibrium was observed between pairs of loci after Bonferroni correction. Tests on deviation from HWE showed a significant excess of heterozygotes only for the *Lf* Dor D 2010 population after correction (Table 1). Average allelic richness (based on 14 samples) and average expected heterozygosity were significantly higher (15 000 permutations, $P < 0.01$) in *L. fluviatilis* (3.270 and 0.501, respectively) than in *L. planeri* (2.871 and 0.444) (Table 1). *Lampetra planeri* sampled in upstream and downstream parts of the Aa, Hem, Bresle and Oir rivers showed no difference of genetic diversity (two-sided paired Wilcoxon test, $P > 0.05$, Table S3) except for expected heterozygosity in Aa 2014 and allelic richness in Oir 2014, which were both significantly higher downstream (Table S3). In most rivers, the allelic richness was significantly higher in *L. fluviatilis* than in *L. planeri* (two-sided paired Wilcoxon test, $P < 0.05$, and Tables 1 and S3), except in sympatric populations and in the Risle River. The same trend was observed for expected heterozygosity (Table S3).

Genetic structure

Pairwise F_{ST} values ranged from 0 to 0.324 (Tables 2, 3 and S4), with an overall F_{ST} of 0.082 (99% CI = 0.065–0.106). The AMOVA revealed that the percentage of variance among populations of the same species (6.25%) was much higher than between species (1.55%) and the largest part of variance (92.20%) was found within populations. F_{ST} among *L. fluviatilis* populations was significant but much smaller than among *L. planeri* populations: 0.022 and 0.134, respectively (15 000 permutations, $P < 0.001$). *L. planeri* populations sampled upstream and downstream of barriers in the same river (Aa, Hem, Bresle and Oir rivers) were not significantly differentiated ($F_{ST\text{-}Aa} = 0.006$; $F_{ST\text{-}Hem} = 0.008$; $F_{ST\text{-}Bresle} = 0.005$) except in the Oir River ($F_{ST\text{-}Oir\text{-}2011} = 0.031$, $F_{ST\text{-}Oir\text{-}2014} = 0.020$).

Table 2 Pairwise F_{ST} values for sympatric populations (nonsignificant values are grey-coloured, and negative values were set to zero).

Populations	*Lf* Aa 2014	*Lf* Hem 2014	*Lf* Bre 2011	*Lf* Bet 2014	*Lf* Oir 2010	*Lf* Oir 2011	*Lf* Oir 2014	*Lp* Aa 2014	*Lp* Hem 2014	*Lp* Bre 2011	*Lp* Bet 2014	*Lp* Oir 2010	*Lp* Oir 2011
Lf Hem D 2014	0												
Lf Bre D 2011	0	0											
Lf Bet D 2014	0.002	0.001	0.003										
Lf Oir D 2010	0.013	0.023	0.021	0.024									
Lf Oir D 2011	0.001	0.001	0.002	0.017	0.018								
Lf Oir D 2014	0	0	0	0	0.018	0							
Lp Aa D 2014	0.080	0.083	0.092	0.076	0.074	0.102	0.083						
Lp Hem D 2014	0.083	0.081	0.082	0.094	0.087	0.074	0.062	0.112					
Lp Bre D 2011	0.081	0.092	0.074	0.111	0.089	0.077	0.087	0.185	0.187				
Lp Bet D 2014	0.022	0.025	0.018	0.028	0.034	0.015	0.027	0.110	0.093	0.080			
Lp Oir D 2010	0.080	0.087	0.080	0.086	0.048	0.073	0.071	0.091	0.12	0.115	0.072		
Lp Oir D 2011	0.030	0.041	0.025	0.027	0.033	0.032	0.029	0.078	0.122	0.090	0.034	0.032	
Lp Oir D 2014	0.013	0.019	0.013	0.018	0.019	0.013	0.008	0.082	0.075	0.072	0.031	0.035	0.019

We observed contrasting levels of population differentiation between *L fluviatilis* and *L. planeri* depending on rivers (Tables 2 and 3). The sympatric population pair in the Oir (2014) was not significantly differentiated (F_{ST} = 0.008), whereas a moderate structuration was observed in the Aa, Hem, Oir (2010 and 2011), Béthune and Bresle (2011) rivers (F_{ST} = 0.080; 0.081; 0.048; 0.032; 0.028 and 0.074, respectively, Table 2). F_{ST} was generally higher in parapatry with population pairs from the Odon, Loire–Cens and Garonne–Saucats rivers being the most differentiated (Table 3). The parapatric population pair from the Risle River was an exception with a low F_{ST} of 0.028 and 0.036 in 2011 and 2014, respectively (Table 3). Overall, F_{ST} between *L. planeri* and *L. fluviatilis* from the same river system was always smaller than the mean F_{ST} between the *L. planeri* population from this river and all other *L. planeri* populations (Fig. 3). Similarly, the mean F_{ST} between a given *L. planeri* population and all other *L. fluviatilis* populations was always smaller than among *L. planeri* populations (Fig. 3).

A positive correlation between genetic and geographic distances was found in the global data set ($r_{Spearman}$ = 0.27, P = 0.004), but when each species was considered separately, the pattern of IBD was stronger in *L. fluviatilis* ($r_{Spearman}$ = 0.79, $P < 0.001$) than in *L. planeri* ($r_{Spearman}$ = 0.40, P = 0.005).

Bayesian clustering analyses

Results from Structure for each population pair illustrated a continuum of differentiation from apparent panmixia in some rivers to strong differentiation in other cases (Fig. 4). In the Oir, Béthune (sympatry) and Risle (parapatry) rivers, all individuals were assigned to both clusters, hence suggesting that *L. planeri* and *L. fluviatilis* formed a single population. However, in the three other sympatric situations (Aa, Hem and Bresle) where the differentiation was higher, two clusters were observed, but some individuals were assigned to the cluster of the other species. In the parapatric Loire–Cens, Dordogne–Garonne–Saucats and Dordogne–Garonne–Jalle de Tiquetorte systems, both species clustered in two groups and very few individuals were assigned to the cluster of the other species. Interestingly, the few *L. planeri* individuals (n = 3) captured in sympatry on the Risle and Odon rivers were mostly assigned to the cluster of *L. fluviatilis* (Figs 4 and S1a–c).

Results from Structure based on the 1023 individuals showed the highest likelihood for k = 8 and 10 (Table S5a). The best Δk values were observed at k = 3, 6 and 8 (Table S5a). We also investigated k = 2 to illustrate the level of admixture between both species (Fig. S1a). At this clustering level, all individuals had mixed membership proportions between the two species. When considering k = 8 (Fig. S1b), we found two widely admixed clusters for *L. fluviatilis*: the first one included samples from the Atlantic coast and the second one those from the English Channel area. The six other clusters included *L. planeri* samples: four clusters corresponded each to one river (the Aa, Hem, Jalle de Tiquetorte and upstream part of the Oir River), the fifth cluster gathered samples from the Odon and Garonne–Saucats rivers, and the sixth cluster included samples from the Bresle and Loire–Cens rivers. *L. planeri* samples from the Oir, Risle and Béthune were strongly admixed with *L. fluviatili*s populations. Analysing species separately confirmed the existence of two clusters for *L. fluviatilis* (Table S5b and Fig. S1d). For *L. planeri*, the most probable number of cluster was k = 9, and each population formed a distinct cluster except the Béthune and Risle, which clustered together (Table S5c and Fig. S1e).

Estimates of recent migration rates within the different population pairs obtained with BayesAss revealed an asymmetric pattern when considering the whole

Table 3 Pairwise F_{ST} values for parapatric populations (nonsignificant values are grey–coloured, and negative values were set to zero).

Populations	*Lf* Aa 2014	*Lf* Hem 2014	*Lf* Bre 2011	*Lf* Ris 2011	*Lf* Ris 2014	*Lf* Odo 2011	*Lf* Oir 2011	*Lf* Oir 2014	*Lf* Loi 2010	*Lf* Loi 2011	*Lf* Dor 2010	*Lf* Dor 2011	*Lf* Gar 2011	*Lp* Aa 2014	*Lp* Hem 2014	*Lp* Bre 2011	*Lp* Ris 2011	*Lp* Ris 2014	*Lp* Odo 2011	*Lp* Oir 2011	*Lp* Oir 2014	*Lp* Cen 2011	*Lp* Jal 2011
Lf Hem D 2014	0																						
Lf Bre D 2011	0	0																					
Lf Ris D 2011	0	0	0																				
Lf Ris D 2014	0	0	0.002	0																			
Lf Odo D 2011	0	0	0.006	0	0																		
Lf Oir D 2011	0.013	0.001	0	0.006	0.004	0.009																	
Lf Oir D 2014	0	0	0	0	0	0.001	0																
Lf Loi D 2010	0.024	0.034	0.015	0.028	0.031	0.033	0.025	0.026															
Lf Loi D 2011	0.026	0.043	0.026	0.027	0.031	0.035	0.032	0.030	0.008														
Lf Dor D 2010	0.059	0.068	0.056	0.055	0.058	0.063	0.065	0.061	0.033	0.014													
Lf Dor D 2011	0.086	0.102	0.101	0.092	0.086	0.091	0.094	0.091	0.072	0.032	0.011												
Lf Gar D 2011	0.037	0.051	0.049	0.037	0.039	0.046	0.046	0.044	0.036	0.003	0.010	0.020											
Lp Aa U 2014	0.091	0.098	0.099	0.100	0.093	0.102	0.112	0.094	0.090	0.082	0.119	0.126	0.082										
Lp Hem U 2014	0.076	0.074	0.072	0.072	0.074	0.060	0.067	0.06	0.078	0.081	0.119	0.129	0.096	0.089									
Lp Bre U 2011	0.087	0.107	0.076	0.099	0.099	0.112	0.082	0.091	0.056	0.085	0.095	0.138	0.130	0.180	0.172								
Lp Ris U 2011	0.027	0.031	0.030	0.028	0.025	0.035	0.021	0.025	0.039	0.041	0.076	0.106	0.042	0.114	0.098	0.089							
Lp Ris U 2014	0.033	0.036	0.035	0.028	0.036	0.031	0.028	0.033	0.048	0.042	0.075	0.108	0.047	0.122	0.094	0.097	0						
Lp Odo U 2011	0.162	0.185	0.178	0.169	0.171	0.187	0.174	0.173	0.179	0.161	0.210	0.228	0.179	0.207	0.226	0.239	0.132	0.151					
Lp Oir U 2011	0.094	0.096	0.090	0.104	0.101	0.114	0.081	0.088	0.083	0.087	0.121	0.142	0.100	0.097	0.098	0.146	0.114	0.116	0.183				
Lp Oir U 2014	0.054	0.056	0.045	0.059	0.061	0.073	0.052	0.045	0.052	0.071	0.107	0.150	0.096	0.083	0.087	0.102	0.079	0.081	0.164	0.013			
Lp Cen U 2011	0.161	0.182	0.159	0.187	0.183	0.190	0.163	0.173	0.136	0.155	0.173	0.203	0.203	0.243	0.246	0.164	0.173	0.161	0.273	0.222	0.204		
Lp Jal U 2011	0.053	0.073	0.059	0.061	0.071	0.066	0.065	0.066	0.064	0.070	0.086	0.12	0.102	0.142	0.136	0.108	0.103	0.094	0.275	0.149	0.113	0.220	
Lp Sau U 2011	0.124	0.113	0.126	0.117	0.12	0.137	0.138	0.135	0.167	0.157	0.192	0.228	0.189	0.186	0.196	0.275	0.162	0.149	0.194	0.181	0.156	0.324	0.210

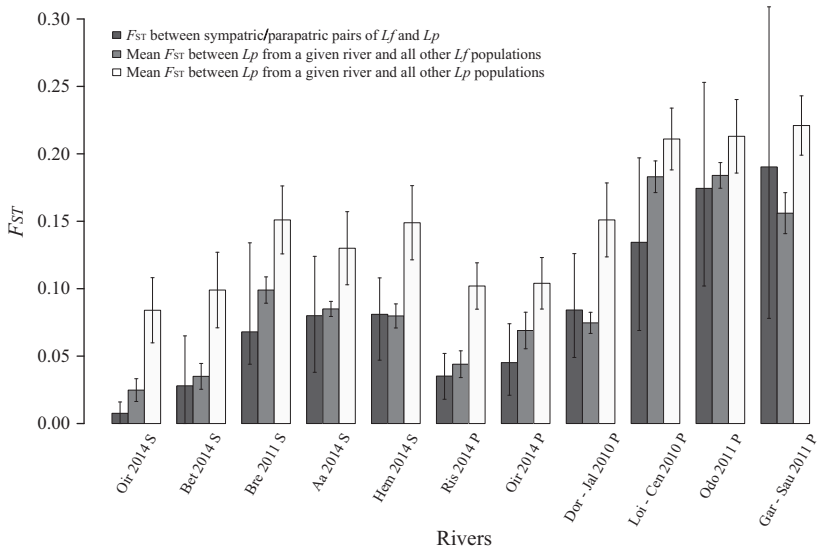

Fig. 3 Comparison of F_{ST} values between sympatric (S) and parapatric (P) pairs of river and brook lampreys (dark-grey bars with their 95% confidence intervals), brook lampreys from a given river and all other brook lamprey populations surveyed (grey bars ± SE) and brook lampreys from a given river and all other river lamprey populations (white bars ± SE).

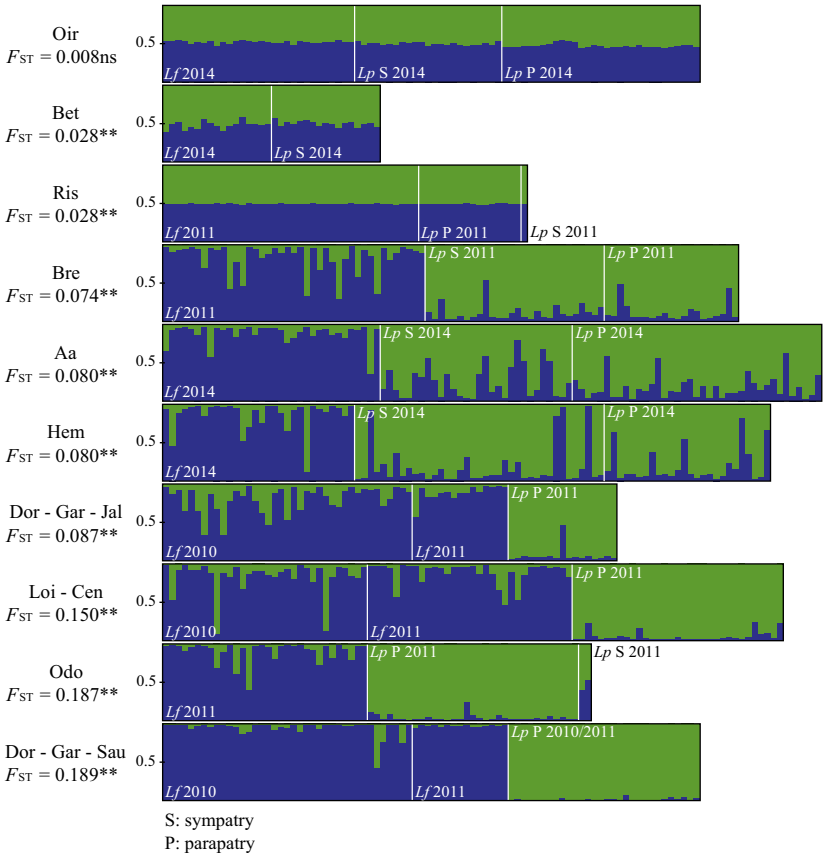

Fig. 4 Bayesian analysis performed with STRUCTURE for each sympatric (S) or parapatric (P) pair of river (*Lf*) and brook (*Lp*) lamprey populations.

data set with a significant tendency for a higher gene flow from *L. planeri* to *L. fluviatilis* (two-sided paired permutations test, $T = 78$, $P = 0.015$) (Table S6). Interestingly, this pattern was driven by asymmetric migration occurring mainly in parapatric situations ($T = 45$, $P = 0.006$), whereas there was no significant difference in the direction of migration in sympatry ($T = 3$, $P = 0.343$).

Discussion

The main aim of our study was to investigate the level of reproductive isolation between two lamprey species by combining experimental measurements of reproductive barriers and analyses of gene flow between sympatric and parapatric population pairs. Our experiments demonstrated that brook lamprey males could reproduce with river lamprey females under semi-natural conditions despite the important size difference between species. Results from artificial fertilizations further supported a low level of reproductive isolation. Population genetic analyses of replicated pairs revealed a continuum of gene flow between species with a pattern of panmixia in some sympatric populations, a moderate differentiation in some other sympatric sites and a strongly reduced gene flow between populations separated by anthropogenic barriers. This gradient of divergence suggests ongoing gene flow in certain sympatric population pairs and some degree of reproductive isolation in other sites. However, secondary contacts after a period of allopatry could also explain this variable degree of genetic differentiation among sympatric sites. In addition, anthropogenic barriers strongly restrict the level of gene flow between species and may thus ultimately promote the evolution of reproductive isolation.

Using artificial fertilizations, we found that *L. planeri* and *L. fluviatilis* males have the same capacity to fertilize eggs of *L. fluviatilis* females. Hatching survival of larvae was also high (nearly 100%) and identical regardless of the male's species. Using a genetically distinct female population (F_{ST} = 0.055), we found that hatching rates were as strong as with crosses using females from the same population, further suggesting a low post-zygotic isolation and no outbreeding depression. Recently, Hume *et al.* (2013a) also observed viable hybrids using a similar experimental approach, but they obtained much lower values of survival potentially because their experimental design did not allow a distinction of unfertilized and dead embryos. Viable hybrid crosses have been obtained between different pairs of lamprey species, but they have always been raised only for a few weeks due to the difficulty of rearing juvenile lampreys (Weissenberg, 1925; Piavis *et al.*, 1970; Beamish & Neville, 1992; Hume *et al.*, 2013a). As a result, the fitness of hybrids has never been thoroughly assessed and was only limited to the F1 generation, which prevents an accurate assessment of intrinsic post-zygotic barriers. Indeed, genetic incompatibilities are generally best revealed in F2s or backcrosses, whereas heterosis is expected in the F1 generation (e.g. Edmands, 1999; Wiley *et al.*, 2009). For instance, Bierne *et al.* (2002, 2006) found a pattern of heterosis in F1 crosses of *Mytilus edulis* and *Mytilus galloprovincialis*, whereas F2s were selected against at the larval stage. As a consequence, further studies are needed to better understand the potential mechanisms of post-zygotic isolation in lampreys.

Even when post-zygotic isolation is low, premating barriers can contribute to reproductive isolation (e.g. Sobel & Streisfeld, 2015). Our results based on mating trials under semi-natural conditions showed for the first time that *L. planeri* males were able to fertilize *L. fluviatilis* females despite the important size difference between species. This interbreeding produced viable hybrid larvae, which suggested a low level of premating isolation and confirmed the low post-zygotic isolation at an early developmental stage observed with *in vitro* fertilizations. Size-assortative mating has been suggested to promote divergence and partial or complete reproductive isolation in many taxa including seahorses (Jones *et al.*, 2003), sticklebacks (McKinnon *et al.*, 2004) or water striders (Han *et al.*, 2010). A similar process has been suggested to induce reproductive isolation in lampreys (Beamish & Neville, 1992), a hypothesis that was not confirmed by our results. However, in our experiment the reproductive success of *L. planeri* males was much lower than the one of *L. fluviatilis* males (Fig. 2); hence, some degree of size-assortative mating may occur. *L. planeri* males may also adopt a sneaking strategy, in which they would fertilize some eggs of a *L. fluviatilis* female despite its tendency to mate with larger conspecific males (Hume *et al.*, 2013b). This tactic is widespread in many fish species and may thus limit the evolution of prezygotic isolation in species pairs of lampreys (Gross, 1984; Gage *et al.*, 1995; Fleming, 1996). However, in the absence of *L. planeri* females in our experiment, *L. planeri* males may have been somehow 'forced' to mate with interspecific females, which may have led to an underestimation of the strength of prezygotic barriers. Further experiments including males and females from both species are thus required to produce quantitative estimates of prezygotic isolation in this system.

The low reproductive isolation measured in our experiments on individuals from a single river (Oir) was mirrored by high levels of gene flow in this sympatric site. However, by studying a total of 10 population pairs, we found a gradient of increasing differentiation with some sympatric pairs forming a genetically homogeneous population, some others being significantly differentiated and parapatric pairs displaying a high level of divergence. Such a gradient of divergence across multiple pairs in sympatry suggests variable levels of reproductive isolation within a single species complex, which has been observed in relatively few systems (e.g. Gagnaire *et al.*, 2013; Powell *et al.*, 2013) and emphasizes the interest of using lampreys as a model in speciation studies.

Sympatric pairs in the Oir and Béthune rivers were not (or weakly) genetically differentiated demonstrating that gene flow can be high between resident and migratory lampreys as suggested in earlier studies (Schreiber & Engelhorn, 1998; Espanhol *et al.*, 2007; Blank *et al.*, 2008; Bracken *et al.*, 2015). Similarly, a

low differentiation has been observed between ecotypes of resident and migratory in rainbow trout *Oncorhynchus mykiss* (e.g. Docker & Heath, 2003) and is also well documented in brown trout *Salmo trutta* (e.g. Hindar *et al.*, 1991; Cross *et al.*, 1992; Pettersson *et al.*, 2001; Charles *et al.*, 2005). This suggests that *L. planeri* and *L. fluviatilis* may also represent two ecotypes of a single species. However, we found other sympatric situations (Aa, Hem and Bresle rivers) where the two species were moderately but significantly differentiated. Accordingly, Mateus *et al.* (2013) found a strong differentiation (F_{ST} = 0.37) in a population pair sampled in the same river system in Portugal. These pairs may be a step further along the divergence continuum, which suggests that disruptive selection and other isolating factors may act in these systems. For instance, some temporal isolation during the spawning season and patchiness of breeding habitat may contribute to ecological divergence between the two species. Analogously, temporal and spatial differences in spawning were linked to genetic differentiation between ecotypes or subpopulations of various salmonid species (Deiner *et al.*, 2007; Pearse *et al.*, 2009). In addition, the magnitude of size differences between species seems to vary among rivers (Table 1) and this factor may contribute to variations of reproductive isolation as predicted by theory (Bolnick, 2011) and observed in other taxa (Arnqvist *et al.*, 1996; McKinnon *et al.*, 2004; Martin, 2013). Our experiments showed a low premating isolation in lampreys from the Oir River where the size difference and the genetic differentiation are low between species. Similar experiments in other sympatric sites would thus be required to better understand the role of size-assortative mating in the evolution of reproductive isolation. Besides, the significant genetic differentiation observed in sympatric situations may also reflect some genetic barriers to gene flow (Wu, 2001; Turner *et al.*, 2005). However, if they exist, these barriers may not be distributed over large portions of the genome and may not efficiently counteract gene flow as genetic differentiation and overall reproductive isolation were low (Barton & Bengtsson, 1986; Wu, 2001).

The continuum of genetic differentiation observed in sympatric sites could arise from two different historical scenarios of divergence: (1) ecologically based speciation with gene flow or (2) differential introgression following a secondary contact after a period of allopatric divergence. If the populations have diverged in allopatry for a period of time too short to allow complete reproductive isolation, it is also possible that secondary contacts have occurred at different times in different areas so that in some cases (e.g. Oir River) a single panmictic population is currently found, whereas in other situations the genome swamping between *L. planeri* and *L. fluviatilis* is still incomplete. Such scenarios of secondary contacts have been suggested in many taxa including Cameroon crater lake cichlids (Martin *et al.*, 2015), whitefish (Gagnaire *et al.*, 2013) and voles (Beysard & Heckel, 2014) and may have also played a crucial role in the evolution of reproductive isolation in the apple maggot, which is considered as a classical model of sympatric speciation (Feder *et al.*, 2003). Nevertheless, cases of ongoing gene flow in sympatry between closely related species have been often interpreted as evidences for ecological speciation, whereas the hypothesis of admixture following a secondary contact (or the one of local adaptation) was either not considered or could not be definitively rejected (Via, 2001; Michel *et al.*, 2010; Hohenlohe *et al.*, 2012; Kautt *et al.*, 2012). These different scenarios of divergence are difficult to disentangle, but new modelling approaches may help tackle this issue as described in several recent studies (Duvaux *et al.*, 2011; Roux *et al.*, 2013, 2014; Butlin *et al.*, 2014; Tine *et al.*, 2014).

In contrast to sympatric situations, we observed high levels of differentiation in most parapatric sites as expected under the joint effects of IBD and anthropogenic barriers to migration. In these situations, migration was reduced and asymmetric from *L. planer*i (upstream) to *L. fluviatili*s (Table S6), highlighting the low migratory ability of *L. fluviatili*s in the presence of obstacles (Russon *et al.*, 2011; Foulds & Lucas, 2013; Bracken *et al.*, 2015). Combined negative effects of distance and barriers on gene flow have also been observed in several fish species (Thrower *et al.*, 2004; Raeymaekers *et al.*, 2008; Gomez-Uchida *et al.*, 2009) and more generally in many taxa (see Templeton *et al.*, 2001; Fahrig, 2003). Our results thus highlight the importance of untangling the effects of habitat fragmentation inducing restricted gene flow from ecological divergence between habitats when studying speciation. Inferences about the speciation process might be obscured by the effect of barriers to migration and IBD. Sampling should thus be carefully designed to clearly distinguish sympatric and parapatric sites even at a within-river scale. Ultimately, habitat fragmentation could promote the evolution of reproductive isolation and lead to founder-induced speciation, but it seems more likely to induce local extinctions (Templeton, 1980, 2008).

Anthropogenic barriers to migration did not have the same impact on patterns of genetic diversity and differentiation of *L. planer*i and *L. fluviatili*s populations. We found high levels of genetic structure combined with clustering at the river level in *L. planeri* suggesting that each resident population tends to evolve as an independent evolutionary unit due to low dispersal ability and isolation by anthropogenic barriers in upstream reaches. Similar results were obtained in earlier studies based on allozyme or mtDNA in northern Europe and in the Iberian Peninsula (Schreiber & Engelhorn, 1998; Pereira *et al.*, 2010) and also recently based on microsatellite data in United Kingdom (Bracken *et al.*, 2015). In contrast, populations of the migratory *L. fluviatilis* were weakly differentiated and structured at the regional level. The genetic diversity (both allelic richness and expected heterozygosity) of *L. planeri* populations was

also lower than the one of *L. fluviatilis* populations indicating an important role of genetic drift in isolated brook lamprey populations. A similar pattern has been observed in several salmonid species between freshwater-resident and anadromous populations (Gomez-Uchida *et al.*, 2009; Perrier *et al.*, 2013). Finally, the fact that IBD was lower in *L. planeri* than in *L. fluviatilis* further highlights the impact of anthropogenic barriers on the genetic differentiation among *L. planeri* populations (see also Bracken *et al.*, 2015).

To conclude, our results suggest that *L. fluviatilis* and *L. planeri* may form partially reproductively isolated ecotypes. The variable levels of gene flow among sympatric sites show that different pairs of populations are either at different stages of divergence along the speciation continuum or at different levels of fusion following secondary contacts. In the first hypothesis, size-assortative mating and selection could act together to maintain phenotypic differences in the face of gene flow. The relative strength of these factors may vary among rivers, resulting in variable progress towards sympatric speciation or even stalled speciation (Bolnick, 2011). In the second hypothesis, similar patterns of varying levels of divergence would arise by secondary contacts (Bierne *et al.*, 2013). Combining genome-wide analyses and modelling of complex historical processes may help untangling these different scenarios. Ultimately, experimental approaches testing the long-term fitness of F1s and later-generation hybrids will allow deeper investigations of the mechanisms of post-zygotic reproductive isolation in this system. Common garden experiments may also allow clarifying the relative roles of phenotypic plasticity and genetic factors in the emergence of parasitic and nonparasitic life histories.

Acknowledgments

We thank F. Marchand, J. Tremblay, A. Oger, V. Dolo, Y. Salaville, R. Lemasquerier, V. Lauronce, C. Taverny, B. Rigault C. Rigaud, Y. Perraud, C. Perrier, G. Sanson, J.-L. Fagard and P. Domalain who helped us collect the samples. We thank L. Benestan and J.-S. Moore for valuable comments on the manuscript as well as D. R. Matute and two anonymous referees. This study was funded by the European Regional Development Fund (transnational programme Interreg IV, Atlantic Aquatic Resource Conservation Project).

References

April, J., Mayden, R.L., Hanner, R.H. & Bernatchez, L. 2011. Genetic calibration of species diversity among North America's freshwater fishes. *Proc. Natl Acad. Sci.* **108**: 10602–10607.

Arnqvist, G., Rowe, L., Krupa, J.J. & Sih, A. 1996. Assortative mating by size: a meta-analysis of mating patterns in water striders. *Evol. Ecol.* **10**: 265–284.

Bank, C., Bürger, R. & Hermisson, J. 2012. The limits to parapatric speciation: Dobzhansky–Muller incompatibilities in a continent–island Model. *Genetics* **191**: 845–863.

Barluenga, M., Stölting, K.N., Salzburger, W., Muschick, M. & Meyer, A. 2006. Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature* **439**: 719–723.

Barrett, R.D.H. & Hoekstra, H.E. 2011. Molecular spandrels: tests of adaptation at the genetic level. *Nat. Rev. Genet.* **12**: 767–780.

Barton, N. & Bengtsson, B.O. 1986. The barrier to genetic exchange between hybridising populations. *Heredity* **57**: 357–376.

Barton, N.H. & Hewitt, G.M. 1985. Analysis of hybrid zones. *Annu. Rev. Ecol. Syst.* **16**: 113–148.

Bates, D., Mächler, M., Bolker, B. & Walker, S. 2014. Fitting linear mixed-effects models using lme4. ArXiv14065823 Stat.

Beamish, R.J. 1987. Evidence that parasitic and nonparasitic life history types are produced by one population of lamprey. *Can. J. Fish Aquat. Sci.* **44**: 1779–1782.

Beamish, R.J. & Neville, C.-E.M. 1992. The importance of size as an isolating mechanism in lampreys. *Copeia* **1992**: 191.

Berner, D., Grandchamp, A.-C. & Hendry, A.P. 2009. Variable progress toward ecological speciation in parapatry: stickleback across eight lake-stream transitions. *Evolution* **63**: 1740–1753.

Beysard, M. & Heckel, G. 2014. Structure and dynamics of hybrid zones at different stages of speciation in the common vole (*Microtus arvalis*). *Mol. Ecol.* **23**: 673–687.

Bierne, N., David, P., Boudry, P. & Bonhomme, F. 2002. Assortative fertilization and selection at larval stage in the mussels *Mytilus edulis* and *M. galloprovincialis*. *Evolution* **56**: 292–298.

Bierne, N., Bonhomme, F., Boudry, P., Szulkin, M. & David, P. 2006. Fitness landscapes support the dominance theory of post-zygotic isolation in the mussels *Mytilus edulis* and *M. galloprovincialis*. *Proc. R. Soc. B Biol. Sci.* **273**: 1253–1260.

Bierne, N., Gagnaire, P.A. & David, P. 2013. The geography of introgression in a patchy environment and the thorn in the side of ecological speciation. *Curr. Zool.* **59**: 72–86.

Blank, M., Jürss, K. & Bastrop, R. 2008. A mitochondrial multigene approach contributing to the systematics of the brook and river lampreys and the phylogenetic position of *Eudontomyzon mariae*. *Can. J. Fish Aquat. Sci.* **65**: 2780–2790.

Bolnick, D.I. 2011. Sympatric speciation in threespine stickleback: why not? *Int. J. Ecol.* **2011**: e942847.

Bracken, F.S.A., Hoelzel, A.R., Hume, J.B. & Lucas, M.C. 2015. Contrasting population genetic structure among freshwater-resident and anadromous lampreys: the role of demographic history, differential dispersal and anthropogenic barriers to movement. *Mol. Ecol.* **24**: 1188–1204.

Butlin, R.K., Saura, M., Charrier, G., Jackson, B., André, C., Caballero, A. *et al.* 2014. Parallel evolution of local adaptation and reproductive isolation in the face of gene flow. *Evolution* **68**: 935–949.

Charles, K., Guyomard, R., Hoyheim, B., Ombredane, D. & Baglinière, J.-L. 2005. Lack of genetic differentiation between anadromous and resident sympatric brown trout (*Salmo trutta*) in a Normandy population. *Aquat. Living Resour.* **18**: 65–69.

Ciereszko, A., Glogowski, J. & Dabrowski, K. 2000. Fertilization in landlocked sea lamprey: storage of gametes, optimal

sperm: egg ratio, and methods of assessing fertilization success. *J. Fish Biol.* **56**: 495–505.

Coyne, J.A. & Orr, H.A. 2004. *Speciation*. W.H. Freeman, Sunderland, MA.

Cross, T.F., Mills, C.P.R. & de Courcy Williams, M. 1992. An intensive study of allozyme variation in freshwater resident and anadromous trout, *Salmo trutta* L., in western Ireland*. *J. Fish Biol.* **40**: 25–32.

Deiner, K., Garza, J.C., Coey, R. & Girman, D.J. 2007. Population structure and genetic diversity of trout (*Oncorhynchus mykiss*) above and below natural and man-made barriers in the Russian River, California. *Conserv. Genet.* **8**: 437–454.

Dey, A., Jeon, Y., Wang, G.-X. & Cutter, A.D. 2012. Global population genetic structure of *Caenorhabditis remanei* reveals incipient speciation. *Genetics* **191**: 1257–1269.

Docker, M.F. 2009. A review of the evolution of nonparasitism in lampreys and an update of the paired species concept. 71–114. In: *Biology, Management, and Conservation of Lampreys in North America* (L.R. Brown, S.D. Chase, M.G. Mesa, R.J. Beamish & P.B. Moyle, eds), pp. 71–114. American Fisheries Society Symposium 72, Bethesda, Maryland.

Docker, M.F. & Heath, D.D. 2003. Genetic comparison between sympatric anadromous steelhead and freshwater resident rainbow trout in British Columbia, Canada. *Conserv. Genet.* **4**: 227–231.

Docker, M.F., Youson, J.H., Beamish, R.J. & Devlin, R.H. 1999. Phylogeny of the lamprey genus *Lampetra* inferred from mitochondrial cytochrome b and ND3 gene sequences. *Can. J. Fish Aquat. Sci.* **56**: 2340–2349.

Docker, M.F., Mandrak, N.E. & Heath, D.D. 2012. Contemporary gene flow between "paired" silver (*Ichthyomyzon unicuspis*) and northern brook (*I. fossor*) lampreys: implications for conservation. *Conserv. Genet.* **13**: 823–835.

Duvaux, L., Belkhir, K., Boulesteix, M. & Boursot, P. 2011. Isolation and gene flow: inferring the speciation history of European house mice. *Mol. Ecol.* **20**: 5248–5264.

Earl, D.A. & vonHoldt, B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **4**: 359–361.

Edmands, S. 1999. Heterosis and outbreeding depression in interpopulation crosses spanning a wide range of divergence. *Evolution* **53**: 1757.

Elias, M., Faria, R., Gompert, Z. & Hendry, A. 2012. Factors influencing progress toward ecological speciation. *Int. J. Ecol.* **2012**: doi:10.1155/2012/235010.

Endler, J.A. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, NJ.

Espanhol, R., Almeida, P.R. & Alves, M.J. 2007. Evolutionary history of lamprey paired species *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) as inferred from mitochondrial DNA variation. *Mol. Ecol.* **16**: 1909–1924.

Estoup, A., Largiader, C.R., Perrot, E. & Chourrout, D. 1996. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Mol. Mar. Biol. Biotechnol.* **5**: 295–298.

Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**: 2611–2620.

Excoffier, L. & Lischer, H.E.L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **10**: 564–567.

Fahrig, L. 2003. Effects of habitat fragmentation on biodiversity. *Annu. Rev. Ecol. Evol. Syst.* **34**: 487–515.

Falush, D., Stephens, M. & Pritchard, J.K. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**: 1567–1587.

Feder, J.L., Berlocher, S.H., Roethele, J.B., Dambroski, H., Smith, J.J., Perry, W.L. *et al.* 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in Rhagoletis. *Proc. Natl Acad. Sci. USA* **100**: 10314–10319.

Felsenstein, J. 1981. Skepticism towards Santa Rosalia, or why are there so few kinds of animals? *Evolution* **35**: 124–138.

Fleming, I.A. 1996. Reproductive strategies of Atlantic salmon: ecology and evolution. *Rev. Fish Biol. Fish* **6**: 379–416.

Foulds, W.L. & Lucas, M.C. 2013. Extreme inefficiency of two conventional, technical fishways used by European river lamprey (*Lampetra fluviatilis*). *Ecol. Eng.* **58**: 423–433.

Gage, M.J.G., Stockley, P. & Parker, G.A. 1995. Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic salmon (*Salmo salar*): theoretical and empirical investigations. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **350**: 391–399.

Gagnaire, P.-A., Pavey, S.A., Normandeau, E. & Bernatchez, L. 2013. The genetic architecture of reproductive isolation during speciation-with-gene-flow in Lake Whitefish species pairs assessed by Rad sequencing. *Evolution* **67**: 2483–2497.

Gaigher, A., Launey, S., Lasne, E., Besnard, A.-L. & Evanno, G. 2013. Characterization of thirteen microsatellite markers in river and brook lampreys (*Lampetra fluviatilis* and *L. planeri*). *Conserv. Genet. Resour.* **5**: 141–143.

Gomez-Uchida, D., Knight, T.W. & Ruzzante, D.E. 2009. Interaction of landscape and life history attributes on genetic diversity, neutral divergence and gene flow in a pristine community of salmonids. *Mol. Ecol.* **18**: 4854–4869.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). http://www.unil.ch/izea/softwares/fstat.html. Updated from Goudet (1995).

Gross, M.R. 1984. Sunfish, salmon, and the evolution of alternatives reproductive strategies and tactics in fishes. In: *Fish Reproduction: Strategies and Tactics* (G. Potts & R.J. Wootton, eds), pp. 55–75. Academic Press, London, England.

Han, C.S., Jablonski, P.G., Kim, B. & Park, F.C. 2010. Size-assortative mating and sexual size dimorphism are predictable from simple mechanics of mate-grasping behavior. *BMC Evol. Biol.* **10**: 359.

Hardisty, M.W. & Potter, I.C. 1971. *The Biology of Lampreys*. Academic Press, New York.

Hendry, A.P. 2009. Ecological speciation! Or the lack thereof? *Can. J. Fish Aquat. Sci.* **66**: 1383–1398.

Hewitt, G.M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* **58**: 247–276.

Hewitt, G.M. 2011. Quaternary phylogeography: the roots of hybrid zones. *Genetica* **139**: 617–638.

Hindar, K., Jonsson, B., Ryman, N. & Stahl, G. 1991. Genetic relationships among landlocked, resident, and anadromous Brown Trout, *Salmo trutta* L. *Heredity* **66**: 83–91.

Hohenlohe, P.A., Bassham, S., Currey, M. & Cresko, W.A. 2012. Extensive linkage disequilibrium and parallel adaptive divergence across threespine stickleback genomes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **367**: 395–408.

Huggins, R.J. & Thompson, A. 1970. Communal spawning of brook and river lampreys, *Lampetra planeri* Bloch and *Lampetra fluviatilis* L. *J. Fish Biol.* **2**: 53–54.

Hume, J.B., Adams, C.E., Mable, B. & Bean, C. 2013a. Post-zygotic hybrid viability in sympatric species pairs: a case study from European lampreys. *Biol. J. Linn. Soc.* **108**: 378–383.

Hume, J.B., Adams, C.E., Mable, B. & Bean, C.W. 2013b. Sneak male mating tactics between lampreys (Petromyzontiformes) exhibiting alternative life-history strategies. *J. Fish Biol.* **82**: 1093–1100.

Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* **411**: 302–305.

Jones, A.G., Moore, G.I., Kvarnemo, C., Walker, D. & Avise, J.C. 2003. Sympatric speciation as a consequence of male pregnancy in seahorses. *Proc. Natl Acad. Sci.* **100**: 6598–6603.

Kaeuffer, R., Peichel, C.L., Bolnick, D.I. & Hendry, A.P. 2012. Parallel and nonparallel aspects of ecological, phenotypic, and genetic divergence across replicate population pairs of lake and stream stickleback. *Evolution* **66**: 402–418.

Kalinowski, S.T., Taper, M.L. & Marshall, T.C. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **16**: 1099–1106.

Kautt, A.F., Elmer, K.R. & Meyer, A. 2012. Genomic signatures of divergent selection and speciation patterns in a "natural experiment", the young parallel radiations of Nicaraguan crater lake cichlid fishes. *Mol. Ecol.* **21**: 4770–4786.

Kirkpatrick, M. & Ravigné, V. 2002. Speciation by natural and sexual selection: models and experiments. *Am. Nat.* **159**: S22–S35.

Knebelsberger, T., Dunz, A.R., Neumann, D. & Geiger, M.F. 2015. Molecular diversity of Germany's freshwater fishes and lampreys assessed by DNA barcoding. *Mol. Ecol. Resour.* **15**: 562–572.

Langerhans, R.B., Gifford, M.E. & Joseph, E.O. 2007. Ecological speciation in Gambusia fishes. *Evolution* **61**: 2056–2074.

Lasne, E., Sabatié, M.-R. & Evanno, G. 2010. Communal spawning of brook and river lampreys (*Lampetra planeri* and *L. fluviatilis*) is common in the Oir River (France). *Ecol. Freshw. Fish* **19**: 323–325.

Mallet, J. 2008. Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**: 2971–2986.

Marie Curie SPECIATION Network, Butlin, R., Debelle, A., Kerth, C., Snook, R.R., Beukeboom, L.W. *et al.* 2012. What do we need to know about speciation?. *Trends Ecol. Evol.* **27**: 27–39.

Martin, C.H. 2013. Strong assortative mating by diet, color, size, and morphology but limited progress toward sympatric speciation in a classic example: Cameroon crater lake cichlids. *Evolution* **67**: 2114–2123.

Martin, C.H., Cutler, J.S., Friel, J.P., Dening, C.T., Coop, G. & Wainwright, P.C. 2015. Complex histories of repeated gene flow in Cameroon crater lake cichlids cast doubt on one of the clearest examples of sympatric speciation. *Evolution* **69**: 1406–1422.

Mateus, C.S., Almeida, P.R., Quintella, B.R. & Alves, M.J. 2011. MtDNA markers reveal the existence of allopatric evolutionary lineages in the threatened lampreys *Lampetra fluviatilis* (L.) and *Lampetra planeri* (Bloch) in the Iberian glacial refugium. *Conserv. Genet.* **12**: 1061–1074.

Mateus, C.S., Stange, M., Berner, D., Roesti, M., Quintella, B.R., Alves, M.J. *et al.* 2013. Strong genome-wide divergence between sympatric European river and brook lampreys. *Curr. Biol.* **23**: R649–R650.

McKinnon, J.S., Mori, S., Blackman, B.K., David, L., Kingsley, D.M., Jamieson, L. *et al.* 2004. Evidence for ecology's role in speciation. *Nature* **429**: 294–298.

Michel, A.P., Sim, S., Powell, T.H.Q., Taylor, M.S., Nosil, P. & Feder, J.L. 2010. Widespread genomic divergence during sympatric speciation. *Proc. Natl Acad. Sci.* **107**: 9724–9729.

Nosil, P. 2012. *E*cological Speciation. Oxford Series in Ecology and Evolution. Oxford University Press, Oxford.

Nosil, P., Crespi, B.J. & Sandoval, C.P. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* **417**: 440–443.

Nosil, P., Harmon, L.J. & Seehausen, O. 2009a. Ecological explanations for (incomplete) speciation. *Trends Ecol. Evol.* **24**: 145–156.

Nosil, P., Funk, D.J. & Ortiz-Barrientos, D. 2009b. Divergent selection and heterogeneous genomic divergence. *Mol. Ecol.* **18**: 375–402.

Okada, K., Yamazaki, Y., Yokobori, S. & Wada, H. 2010. Repetitive sequences in the lamprey mitochondrial DNA control region and speciation of *Lethenteron*. *Gene* **465**: 45–52.

Peakall, R. & Smouse, P. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28**: 2537–2539.

Pearse, D.E., Hayes, S.A., Bond, M.H., Hanson, C.V., Anderson, E.C., Macfarlane, R.B. *et al.* 2009. Over the falls? Rapid evolution of ecotypic differentiation in steelhead/rainbow trout (*Oncorhynchus mykiss*). *J. Hered.* **100**: 515–525.

Pereira, A.M., Robalo, J.I., Freyhof, J., Maia, C., Fonseca, J.P., Valente, A. *et al.* 2010. Phylogeographical analysis reveals multiple conservation units in brook lampreys *Lampetra planeri* of Portuguese streams. *J. Fish Biol.* **77**: 361–371.

Perrier, C., Bourret, V., Kent, M.P. & Bernatchez, L. 2013. Parallel and nonparallel genome-wide divergence among replicate population pairs of freshwater and anadromous Atlantic salmon. *Mol. Ecol.* **22**: 5577–5593.

Pettersson, J.C.E., Hansen, M.M. & Bohlin, T. 2001. Does dispersal from landlocked trout explain the coexistence of resident and migratory trout females in a small stream? *J. Fish Biol.* **58**: 487–495.

Piavis, G.W., Howell, J.H. & Smith, A.J. 1970. Experimental hybridization among five species of lampreys from the great lakes. *Copeia* **1970**: 29–37.

Potter, I.C. 1980. Ecology of larval and metamorphosing lampreys. *Can. J. Fish Aquat. Sci.* **37**: 1641–1657.

Powell, T.H.Q., Hood, G.R., Murphy, M.O., Heilveil, J.S., Berlocher, S.H., Nosil, P. *et al.* 2013. Genetic divergence along the speciation continuum: the transition from host race to species in Rhagoletis (Diptera: Tephritidae). *Evolution* **67**: 2561–2576.

Præbel, K., Knudsen, R., Siwertsson, A., Karhunen, M., Kahilainen, K.K., Ovaskainen, O. *et al.* 2013. Ecological speciation in postglacial European whitefish: rapid adaptive

radiations into the littoral, pelagic, and profundal lake habitats. *Ecol. Evol.* **3**: 4970–4986.
Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.
R Development Core Team. 2011. *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
Raeymaekers, J.A.M., Maes, G.E., Geldof, S., Hontis, I., Nackaerts, K. & Volckaert, F.A.M. 2008. Modeling genetic connectivity in sticklebacks as a guideline for river restoration. *Evol. Appl.* **1**: 475–488.
Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223.
Rodríguez-Muñoz, R. & Tregenza, T. 2009. Genetic compatibility and hatching success in the sea lamprey (*Petromyzon marinus*). *Biol. Lett.* **5**: 286–288.
Roesti, M., Hendry, A.P., Salzburger, W. & Berner, D. 2012. Genome divergence during evolutionary diversification as revealed in replicate lake-stream stickleback population pairs. *Mol. Ecol.* **21**: 2852–2862.
Rosenberg, N.A. 2004. DISTRUCT: a program for the graphical display of population structure. *Mol. Ecol. Notes* **4**: 137–138.
Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**: 1219–1228.
Rousset, F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Mol. Ecol. Resour.* **8**: 103–106.
Roux, C., Tsagkogeorga, G., Bierne, N. & Galtier, N. 2013. Crossing the species barrier: genomic hotspots of introgression between two highly divergent *Ciona intestinalis* species. *Mol. Biol. Evol.* **7**: 1574–1587.
Roux, C., Fraïsse, C., Castric, V., Vekemans, X., Pogson, G.H. & Bierne, N. 2014. Can we continue to neglect genomic variation in introgression rates when inferring the history of speciation? A case study in a Mytilus hybrid zone. *J. Evol. Biol.* **27**: 1662–1675.
Russon, I.J., Kemp, P.S. & Lucas, M.C. 2011. Gauging weirs impede the upstream migration of adult river lamprey *Lampetra fluviatilis*. *Fish. Manag. Ecol.* **18**: 201–210.
Salewski, V. 2003. Satellite species in lampreys: a worldwide trend for ecological speciation in sympatry? *J. Fish Biol.* **63**: 267–279.
Schierup, M.H. & Christiansen, F.B. 1996. Inbreeding depression and outbreeding depression in plants. *Heredity* **77**: 461–468.
Schreiber, A. & Engelhorn, R. 1998. Population genetics of a cyclostome species pair, river lamprey (*Lampetra fluviatilis* L.) and brook lamprey (*Lampetra planeri* Bloch). *J. Zool. Syst. Evol. Res.* **36**: 85–99.
Seehausen, O., Butlin, R.K., Keller, I., Wagner, C.E., Boughman, J.W., Hohenlohe, P.A. *et al.* 2014. Genomics and the origin of species. *Nat. Rev. Genet.* **15**: 176–192.
Sobel, J.M. & Streisfeld, M.A. 2015. Strong premating reproductive isolation drives incipient speciation in *Mimulus aurantiacus*. *Evolution* **69**: 447–461.
Sobel, J.M., Chen, G.F., Watt, L.R. & Schemske, D.W. 2010. The biology of speciation. *Evolution* **64**: 295–315.
Soria-Carrasco, V., Gompert, Z., Comeault, A.A., Farkas, T.E., Parchman, T.L., Johnston, J.S. *et al.* 2014. Stick insect genomes reveal natural selection's role in parallel speciation. *Science* **344**: 738–742.
Templeton, A.R. 1980. The theory of speciation via the founder principle. *Genetics* **94**: 1011–1038.
Templeton, A.R. 2008. The reality and importance of founder speciation in evolution. *BioEssays* **30**: 470–479.
Templeton, A.R., Robertson, R.J., Brisson, J. & Strasburg, J. 2001. Disrupting evolutionary processes: the effect of habitat fragmentation on collared lizards in the Missouri Ozarks. *Proc. Natl Acad. Sci. USA* **98**: 5426–5432.
Thrower, F., Iii, C.G., Nielsen, J. & Joyce, J. 2004. A comparison of genetic variation between an anadromous steelhead, *Oncorhynchus mykiss*, population and seven derived populations sequestered in freshwater for 70 Years. *Environ. Biol. Fishes* **69**: 111–125.
Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R.S.T. *et al.* 2014. European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nat. Commun.* **5**: 5770.
Turner, T.L., Hahn, M.W. & Nuzhdin, S.V. 2005. Genomic Islands of Speciation in Anopheles gambiae. *PLoS Biol.* **3**: e285.
Via, S. 2001. Sympatric speciation in animals: the ugly duckling grows up. *Trends Ecol. Evol.* **16**: 381–390.
Vladykov, V.D. & Kott, E. 1979. Satellite species among the holarctic lampreys (Petromyzonidae). *Can. J. Zool.* **57**: 860–867.
Waser, N.M. & Price, M.V. 1985. Reciprocal transplant experiments with *Delphinium nelsonii* (Ranunculaceae): evidence for local adaptation. *Am. J. Bot.* **72**: 1726.
Waser, N.M. & Price, M.V. 1994. Crossing-distance effects in *Delphinium nelsonii*: outbreeding and inbreeding depression in progeny Fitness. *Evolution* **48**: 842.
Weir, B.S. & Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.
Weissenberg, R. 1925. Fluss- und Bachneuunauge (*Lampetra fluviatilis* L. und *Lampetra planeri* Bloch), ein morphologisch-biologischer *Vergleich*. *Zool. Anz.* **63**: 293–306.
Wiley, C., Qvarnström, A., Andersson, G., Borge, T. & Saetre, G.-P. 2009. Postzygotic isolation over multiple generations of hybrid descendents in a natural hybrid zone: how well do single-generation estimates reflect reproductive isolation? *Evolution* **63**: 1731–1739.
Wilson, G.A. & Rannala, B. 2003. Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* **163**: 1177–1191.
Wu, C.-I. 2001. The genic view of the process of speciation. *J. Evol. Biol.* **14**: 851–865.
Yamazaki, Y., Yokoyama, R., Nishida, M. & Goto, A. 2006. Taxonomy and molecular phylogeny of *Lethenteron* lampreys in eastern Eurasia. *J. Fish Biol.* **68**: 251–269.
Zanandrea, G.S.J. 1959. Speciation among Lampreys. *Nature* **184**: 380.
Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. 2009. *Mixed Effects Models and Extensions in Ecology With R*. Springer New York, New York, NY.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Detailed protocol for artificial fertilizations.

Figure S1 Bayesian individual clustering results with STRUCTURE: $k = 2$, 8 and 10 for the two species analysed together (a, b and c, respectively) and $k = 2$ and 9 for *Lampetra fluviatilis* (d) and *Lampetra planeri* (e) respectively.

Table S1 Results of a Generalized Linear Mixed Model (GLMM) testing the effect of cross type (homo- vs. heterospecific) and maternal population (Loire vs. Oir) on fertilization success of *Lampetra fluviatilis* eggs.

Table S2 Genetic diversity estimates for each locus in each population.

Table S3 Results of permutations tests comparing Allelic richness (Ar) and expected heterozygosity (He) between *Lf* and *Lp* in each river system.

Table S4 Pairwise F_{ST} among all populations (non-significant values are grey coloured).

Table S5 Results of STRUCTURE analysis for each dataset: (a) full dataset, (b) *Lampetra fluviatilis* only and (c) *Lampetra planeri* only.

Table S6 Estimates of ongoing migration rates (*m*) obtained with BayesAss (Wilson & Rannala, 2003) from river lampreys to brook lampreys (*m* in *Lp* from *Lf*) and from brook lampreys to river lampreys (*m* in *Lf* from *Lp*).

Data deposited at Dryad: doi: 10.5061/dryad.5qv85

Received 18 February 2015; revised 2 September 2015; accepted 2 September 2015