

## Temporal changes in allele frequencies in a small marble trout *Salmo marmoratus* population threatened by extreme flood events

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(Received 6 July 2015, Accepted 14 December 2015)

The effect of extreme floods on the genetic composition of marble trout *Salmo marmoratus* living in Lipovscek, a tributary of the Soca River in Slovenia, which has been affected by multiple destructive flood events for centuries was investigated. By monitoring genetic variability during the period 2004–2011, apparent signatures of genetic erosion including a decline in observed and expected heterozygosities and allelic richness were observed. Contemporary effective population size was estimated between 11 and 55 individuals, which is congruent with census data. The data suggest asymmetric gene flow between the two sections of the river. The existence of substantial downstream migration (15–19%) was confirmed by paternity analysis. A small (1–3%) upstream migration was also suggested, which was confirmed by tagging data. Overall, low genetic diversity has not prevented the survival of the Lipovscek population, which might be a common feature of salmonid freshwater populations.

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Key words: disturbance events; genetic erosion; genetic events; recurring floods; resilience; salmonids.

### INTRODUCTION

Climate change is predicted to lead to more frequent and intense extreme weather events, such as floods, heat waves or droughts (IPCC, 2012). There are numerous examples in recent scientific literature of increased frequency and intensity of extreme weather events being attributed to human effect on climate change including increase in global dry areas (Dai, 2011), heavy precipitation (Min *et al.*, 2011), floods (Pall *et al.*, 2011), extreme temperatures (Zwiers *et al.*, 2011), heat waves (Coumou & Rahmstorf, 2012) and extreme weather anomalies (Hansen *et al.*, 2012). Despite the growing awareness that a significant fraction of species are at risk of extinction due to extreme events and associated catastrophic disturbances (Parmesan & Yohe, 2003; Thomas *et al.*, 2004; IPCC, 2012), the demographic and evolutionary consequences of extreme events have been poorly explored.

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Stream-dwelling salmonids are good candidates to study the demographic and evolutionary consequences of severe disturbance events as they are commonly susceptible to substantial environmental fluctuations including temporal changes in mean stream flow and extreme events (Grossman *et al.*, 1982, 1998; Lake, 2000). In particular, flash floods and debris flows are probably the most important threats to the viability of small freshwater salmonid populations, along with habitat fragmentation and increased water temperatures (Jensen & Johnsen, 1999; Vincenzi *et al.*, 2008a). Floods can induce episodes of massive mortalities as a result of high water velocities and sediment movement. One of the putative adaptations that may confer resilience to flood events is fast body growth and relaxation of density-dependent early mortality at low densities, which enables surviving individuals to reach sexual maturity at younger ages and populations to quickly recover and increase in numbers when greatly reduced in size by catastrophic events (Jenkins *et al.*, 1999; Roghair *et al.*, 2002; Vincenzi *et al.*, 2008a, 2012a, 2014). This can explain the fast recovery (within few years) to pre-flood conditions observed in many salmonid populations susceptible to catastrophic floods (Lamberti *et al.*, 1991; Propst & Stefferud, 1997; Vincenzi *et al.*, 2008a).

The present model system is the stream-dwelling marble trout *Salmo marmoratus* Cuvier 1829, an endemic fish of the Adriatic Sea basin, found on the alpine part of the River Po in northern Italy, Slovenia, Croatia, Bosnia-Herzegovina and Montenegro (Crivelli *et al.*, 2000; Snoj *et al.*, 2009). *Salmo marmoratus* is at risk of extinction, mostly due to hybridization with non-indigenous brown trout *Salmo trutta* L. 1758 introduced in the rivers through restocking practices (Crivelli, 1995). For example, pure *S. marmoratus* individuals were identified using assignment tests based on microsatellite data in five out of nine sampling sites in the Adige River system in South Tyrol, while hybrids were found at all locations (Meraner *et al.*, 2010). The study of Pujolar *et al.* (2011a) using mitochondrial and microsatellite markers also showed high level of hybridization in the River Po in northern Italy, the Adige River in South Tyrol and the Soca River in Slovenia, although pure non-introgressed *S. marmoratus* populations were found in headwaters of all three river systems.

A conservation programme was launched in 1993 to protect and conserve the genetic and life-history uniqueness of *S. marmoratus* in Slovenia (Crivelli *et al.*, 2000). At present, only seven remnant pure populations of *S. marmoratus* remain in the Adriatic basin of Slovenia, persisting in secluded headwaters above impassable waterfalls. Recurrent major floods and associated debris flows are the principal threats to the persistence of *S. marmoratus* populations living in Slovenian streams (Vincenzi *et al.*, 2008a). Triggered by high-intensity rainfalls in the Slovenian Alps and surrounding areas, major autumnal floods have been recorded since the 18th Century, recurring approximately every 50 years (Zorn *et al.*, 2006). Moreover, a critical increase in the frequency of autumnal floods has been observed in recent years (Vincenzi *et al.*, 2012b) and since 2009 spring floods have been observed in the region (Pujolar *et al.*, 2011b).

The genetic analysis of Slovenian *S. marmoratus* samples has shown a pattern of high genetic differentiation across streams and low genetic diversity within streams (Fumagalli *et al.*, 2002; Pujolar *et al.*, 2011b). The reduced level of genetic variability observed in all populations has been attributed to a strong effect of genetic drift, exacerbated by high mortalities associated with recurrent flash flood and debris floods (Pujolar *et al.*, 2011b). Bayesian demographic analysis is concordant with a scenario of serial bottleneck episodes occurring for 100–800 years (Pujolar *et al.*, 2011b).

Genetic markers have been extensively used to monitor salmonid populations and infer demographic history, including detection of inbreeding and population declines and bottleneck events (Allendorf & Waples, 1996; García-Marín *et al.*, 1999; Wang *et al.*, 2002; Utter, 2005). In this study, the effect of extreme floods and associated debris flows on the demography and genetics of *S. marmoratus* over time was investigated, focusing on the Lipovscek Stream, a tributary of the Soca River in Slovenia. The Lipovscek Stream has experienced the highest occurrence of autumnal floods in the area in the last two decades (Pujolar *et al.*, 2011*b*). The Lipovscek population was monitored during the period 2004–2011 to investigate the effect of the extreme flood events that occurred in 2004, 2007 and 2009 by comparing pre- and post-flood samples and to test for changes in genetic composition and genetic diversity in the population throughout time. Contemporary effective population size was investigated and compared with census data. The possible existence of migration between the upstream and downstream section of the stream was also tested and finally a parentage analysis of all fish present in the stream was investigated. Due to the effect of recurrent floods, it was predicted that the Lipovscek population of *S. marmoratus* harboured low genetic variability and, therefore, possessed a low adaptive potential to environmental changes.

## MATERIALS AND METHODS

### SAMPLING

Genetic samples were obtained from the tag–recapture monitoring programme of the Lipovscek Stream, a tributary of the Baca River within the Soca River system in Slovenia, between 2004 and 2011, where *S. marmoratus* persist above an impassable 5 m high waterfall. The stream is constituted by a downstream section (length: 107.3 m; surface: 352 m<sup>2</sup>; number of pools: 11; altitude: 425.3–432.4 m) and an upstream section (length: 94.3 m; surface: 290 m<sup>2</sup>; number of pools: 3; altitude: 454.5–460.6 m). The two sections are separated by a 3 m high waterfall which is very difficult to pass in an upstream direction except during major floods. Sampling was conducted every June and September on the whole length of the stream. During each sampling survey, the stream was electrofished two times to produce a multiple-pass removal estimate of *S. marmoratus* abundance using Microfish 3.0 (Van Deventer & Platts, 1989). Estimates were made for three total length ( $L_T$ ) classes: 0+>100 mm, immature fish 101–200 mm and >200 mm adult fish. The probability of recapture was >80% and no evidence was found for capture probability varying with age and size of the fish older than age 0 year (Vincenzi *et al.*, 2008*b*). All captured fish aged >1+ year and with a minimum size of 115 mm were anaesthetized with benzocaine, marked with Carlin tags made in Sweden (Institute of Freshwater Research; www.slu.se) and measured for  $L_T$  (to the nearest mm) and mass ( $M$ , g). Age was determined by tag examination or by reading scales if the individual was not tagged (first sampling or tag lost). Scale reading follows standard terminology: *S. marmoratus* in the first year of life were indicated as 0+ year and in the second year, subsequent to winter annulus formation, as 1+ year.

Finclips for genetic analysis were collected from anaesthetized individuals that were immediately released back to the stream. The genetic study included all individuals sampled in the Lipovscek Stream during the period 2008–2011 ( $n = 261$ ). Additionally, the genetic study also included 30 pre-2004 flood and 30 post-2004 flood samples previously analysed by Pujolar *et al.* (2011*b*).

### MICROSATELLITE ANALYSIS

DNA was extracted from ethanol-preserved finclips using the following protocol: minute sections of tissue were digested for 4 h at 56° C in a lysis buffer containing 100 µl of TE buffer,

7  $\mu$ l of dithiothreitol (DTT) and 1  $\mu$ l of proteinase K solution (20 mg ml<sup>-1</sup>). After incubation at 96° C for 10 min, samples were centrifuged for 11 min at maximum speed and the supernatant was stored in a freezer at -20° C.

All individuals were genotyped at a total of 18 microsatellite loci that were polymorphic in *S. marmoratus* populations from Slovenia (Pujolar *et al.*, 2011b). PCR products were obtained in a GeneAmp PCR System 2700 Thermocycler (Applied Biosystems; www.appliedbiosystems.com) using the QIAGEN Multiplex PCR Kit (QIAGEN; www.qiagen.com). PCR conditions are detailed in Pujolar *et al.* (2011b). Microsatellite polymorphism was screened in an ABI 3130 AVANT automatic capillary sequencer (Applied Biosystems).

## DATA ANALYSIS

Allele frequencies and measures of genetic diversity including polymorphism at the 95% ( $P_{95}$ ) and 99% level ( $P_{99}$ ) and observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities were calculated using GENETIX version 4.05 (Belkhir *et al.*, 2005). Allelic richness ( $A_R$ ) was calculated using FSTAT (Goudet, 2002). Differences in genetic diversity among samples were tested using one-way ANOVA in STATISTICA version 6.0 (StatSoft; www.statsoft.com). Deviations from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium and differences in allele frequencies among samples were tested using GENEPOP (Raymond & Rousset, 1995). In all cases, significance levels were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice, 1989). Presence of null alleles was tested using the programme MICROCHECKER version 2.2.3 (Van Oosterhout *et al.*, 2004).

Population structure was studied using non-hierarchical and hierarchical  $F$ -statistics (Weir & Cockerham, 1984) calculated using ARLEQUIN ver 3.0 (Excoffier & Lischer, 2010). Genetic variability was partitioned into a temporal (across sampling years) and a geographic (upstream *v.* downstream section) component using the 'locus-by-locus AMOVA' option in ARLEQUIN. Significance tests were assessed with 10 000 permutation tests. A principal component analysis (PCA) was conducted using the function *princomp* in R based on genetic distances between sample pairs using  $F_{ST} (1 - F_{ST})^{-1}$ .

Effective population size ( $N_e$ ) was estimated using the software NeEstimator v2 (Do *et al.*, 2014). Contemporary  $N_e$  was estimated using the temporal method since the data consists of a single population sampled at several time periods. Three separate temporal (two-sample) estimators were used:  $F_K$  (Pollak, 1983),  $F_C$  (Nei & Tajima, 1981) and  $F_S$  (Jorde & Ryman, 2007). For this analysis, year-of-birth cohort data were employed, using the following generation set: cohort 2005 (generation 0), cohort 2007 (generation 2), cohort 2008 (generation 3), cohort 2010 (generation 5) and cohort 2011 (generation 6). Estimates were calculated for all possible pair-wise comparisons with cohort 2011, which includes the largest number of individuals.

Exchange of individuals between the downstream and upstream sections of the stream was estimated using BayesAss version 3.0 (Wilson & Rannala, 2003). The programme conducts a Bayesian inference of recent migration rates using multilocus genotypes. Migration rates were calculated from upstream to downstream and from downstream to upstream using the 2011 sample, which is the largest sample in the study ( $n = 222$ ). The method assumes relatively low levels of migration and the proportions of migrant individuals into a population cannot exceed one third of the population total each generation. Parameters used for the Markov chain Monte Carlo (MCMC) included a total of 30 million iterations, a sampling frequency of 2000 and a 10% burn-in. Two separate runs were conducted for each analysis.

Paternity was assessed using CERVUS v. 3.0 (Kalinowski *et al.*, 2007), which implements a simulation procedure to infer the most likely parents. Individuals from the cohort 2011 were assigned to the candidate parents using the genotypes of all adults present at the river in the previous year. Only parent pair assignments with confidence levels of 95% were considered. Moreover, pair-wise relatedness among all individuals from the 2011 cohort ( $n = 197$ ) was inferred using the software COLONY (Wang, 2004). The programme searches for the maximum likelihood partition of a sample of individuals into full-sib and half-sib clusters, sharing both or one parent, respectively. COLONY was run under the assumption of polygamy mating, as it has been shown in salmonids (Kanno *et al.*, 2011). Additional scenarios (monogamy and only one parent polygamous) were also tested. Full sibs and half sibs were identified at  $P > 0.95$ . The programme was run separately for the two sections (upstream and downstream) of the river.

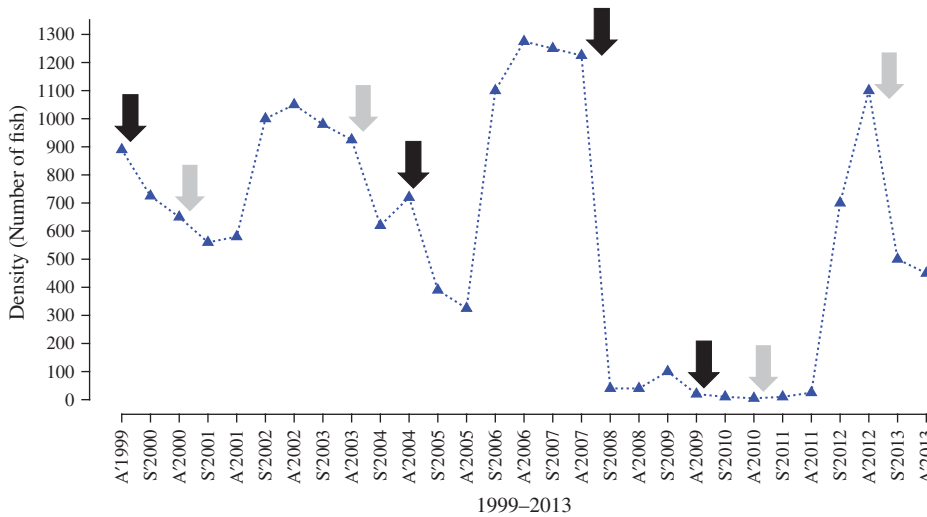


Fig. 1. Estimated number of *Salmo marmoratus* individuals aged >1+ year in the Lipovscek Stream from 1999 to 2013 (A, autumn; S, spring) based on removal–depletion of sampling data. Major (➡) and medium (➡) floods are indicated.

## RESULTS

### POPULATION TREND

Details on the abundance trend of *S. marmoratus* individuals older than age 1 year (individuals aged >1+ year) in the Lipovscek Stream during the period 1999–2013 based on the removal of sampling data are given in Fig. 1. The Lipovscek population showed good signs of recovery after a major autumnal flood in 2004, but in September 2007 a new major flood occurred that killed 92.4% of the population, leaving only 38 fish aged >1+ year. Following another major flood in 2009 and a moderate flood in 2010, the population was reduced to only 10 fish aged >1+ year in the whole stream. Sampling in 2011, however, showed a recovery of abundance levels with the presence of 15 individuals aged 1+ year from the 2010 cohort and 197 individuals aged 0+ year from the 2011 cohort.

Tagging data showed how most individuals persisted in the stream in periods with no floods and for instance 88.2% of fish aged >+1 year present in September 2008 were recaptured in June 2009. By contrast, only 23.8% of fish aged >+1 year were recaptured following the major autumnal flood in 2009. Overall, the severity of the floods is apparent as only two fish aged >+1 year tagged in 2008 were still present in the stream in September 2011. All fish were recaptured in the same section of the stream in which they were originally tagged except for one individual caught in the downstream sector in June 2008 and in the upstream sector in September 2008.

### GENETIC DIVERSITY

A total of nine of the 18 loci were polymorphic (Table I). Overall genetic diversity was low and many loci showed a discontinuous allele distribution, with missing alleles across the allele size range and few or no rare alleles. Comparison of genetic diversity

TABLE I. Allele frequencies and measures of genetic diversity at 18 loci for (a) all samples, (b) year-of-birth cohorts and (c) samples from upstream and downstream sections, including observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities, polymorphism at the 95% level ( $P_{95}$ ), mean (MNA) and total (TNA) number of alleles and allelic richness ( $A_R$ ), and sample size ( $n$ )

(a) Samples	2004 ( $n = 30$ )	2005 ( $n = 30$ )	2008 ( $n = 48$ )	2010 ( $n = 26$ )	2011 ( $n = 222$ )
$H_O$	0.141	0.110	0.086	0.064	0.076
$H_E$	0.116	0.092	0.096	0.073	0.074
$P_{95}$	0.333	0.278	0.333	0.167	0.222
MNA	1.67	1.44	1.72	1.50	1.61
TNA	30	26	31	27	29
$A_R$	1.62	1.43	1.60	1.47	1.46

(b) Cohorts	Cohort 2005 ( $n = 8$ )	Cohort 2006 ( $n = 5$ )	Cohort 2007 ( $n = 10$ )	Cohort 2008 ( $n = 10$ )	Cohort 2010 ( $n = 16$ )	Cohort 2011 ( $n = 197$ )
$H_O$	0.063	0.089	0.072	0.067	0.076	0.077
$H_E$	0.077	0.076	0.076	0.099	0.074	0.074
$P_{95}$	0.333	0.167	0.278	0.333	0.167	0.222
MNA	1.44	1.22	1.44	1.44	1.28	1.56
TNA	26	22	26	26	23	28
$A_R$	1.31	1.22	1.29	1.34	1.20	1.26

(c) Sections	Downstream 2008 ( $n = 24$ )	Downstream 2010 ( $n = 19$ )	Downstream 2011 ( $n = 128$ )	Upstream 2008 ( $n = 24$ )	Upstream 2010 ( $n = 7$ )	Upstream 2011 ( $n = 94$ )
$H_O$	0.081	0.076	0.071	0.090	0.032	0.081
$H_E$	0.085	0.080	0.071	0.097	0.043	0.077
$P_{95}$	0.167	0.167	0.222	0.333	0.278	0.278
MNA	1.72	1.39	1.56	1.61	1.28	1.39
TNA	31	25	28	29	23	25
$A_R$	1.35	1.28	1.30	1.38	1.28	1.26

values at 18 loci during the period 2004–2011 showed evidence of genetic erosion in the Lipovscek population (Table I), with a large drop in observed (from 0.141 in 2004 to 0.076 in 2011), and expected heterozygosities (from 0.116 in 2004 to 0.074 in 2011). While mean and total number of alleles did not drop significantly (30 in 2004 and 29 in 2011), allelic richness decreased from 1.64 in 2004 to 1.46 in 2011. During the period 2004–2011, genetic diversities showed large fluctuations (Table I), with the lowest values observed in 2010 and an overall increase in 2011 relative to 2010.

Cohort analysis (2005–2011) showed similar diversity values across cohorts (Table I). Surprisingly, the 2011 cohort showed similar values to the rest of the cohorts despite presenting a much larger population size ( $n = 197$ ) in comparison with the rest of the cohorts ( $n = 5–16$ ). Concordantly, the 2011 cohort presented the largest mean and total number of alleles but an average allelic richness.

Comparison of genetic diversities across the two sections of the river showed (on average) higher variability on the upstream part of the river (Table I), except for the 2010 sample (related to its small sample size,  $n = 7$ ). Taking into account the 2011 samples, the upstream part of the river showed higher values of observed ( $H_O = 0.081$ ) and expected ( $H_E = 0.077$ ) heterozygosities than the downstream part of the river ( $H_O = 0.071$ ,  $H_E = 0.071$ ), but not significantly.

## GENETIC DIFFERENTIATION

Comparison of allele frequencies during the period 2004–2011 showed significant differences at two loci (*Str543*:  $P < 0.001$ ; *Str85*:  $P < 0.001$ ), at which frequencies of the most common allele increased from 0.52 to 0.78 and from 0.56 to 0.81, respectively. The 2004 and 2005 samples clustered together in a PCA, while the 2011 sample appeared as the most differentiated sample [Fig. 2(a)].

Similarly, cohort analysis showed differences at the same two loci (*Str543*:  $P < 0.05$ ; *Str85*:  $P < 0.001$ ). Cohorts 2006, 2007 and 2008 clustered together in a PCA, which showed the cohort 2011 as the most differentiated [Fig. 2(b)].

When comparing the upstream and downstream sections of the river, significant differences were found at four loci (*CA059136*:  $P < 0.001$ ; *Str543*:  $P < 0.001$ ; *Str591*:  $P < 0.01$ ; *Str85*:  $P < 0.01$ ). Upstream and downstream samples did not cluster together in a PCA, where samples generally clustered according to year rather than river section [Fig. 2(c)]. A hierarchical AMOVA partitioning genetic differentiation ( $F_{ST} = 0.082$ ) showed larger differences between temporal samples (*i.e.* across sampling years:  $F_{CT} = 0.057$ ) than geographic samples (*i.e.* upstream *v.* downstream:  $F_{SC} = 0.024$ ).

## EFFECTIVE POPULATION SIZE

Estimates of contemporary effective population size are summarized in Table II. Similar  $N_e$  values were obtained using three different temporal estimators:  $F_K$  ranged between 14.6 and 39.8,  $F_C$  between 13.0 and 54.4 and  $F_S$  between 11.5 and 31.7. Confidence intervals were larger using the  $F_K$  and  $F_C$  estimators, while  $F_S$  showed the smallest confidence intervals, with a maximum  $N_e$  of 63.1.

## ESTIMATION OF MIGRATION RATES

Migration of individuals between the upstream and downstream sections of the Lipovscek Stream was estimated using the individuals sampled in 2011 (Table III).

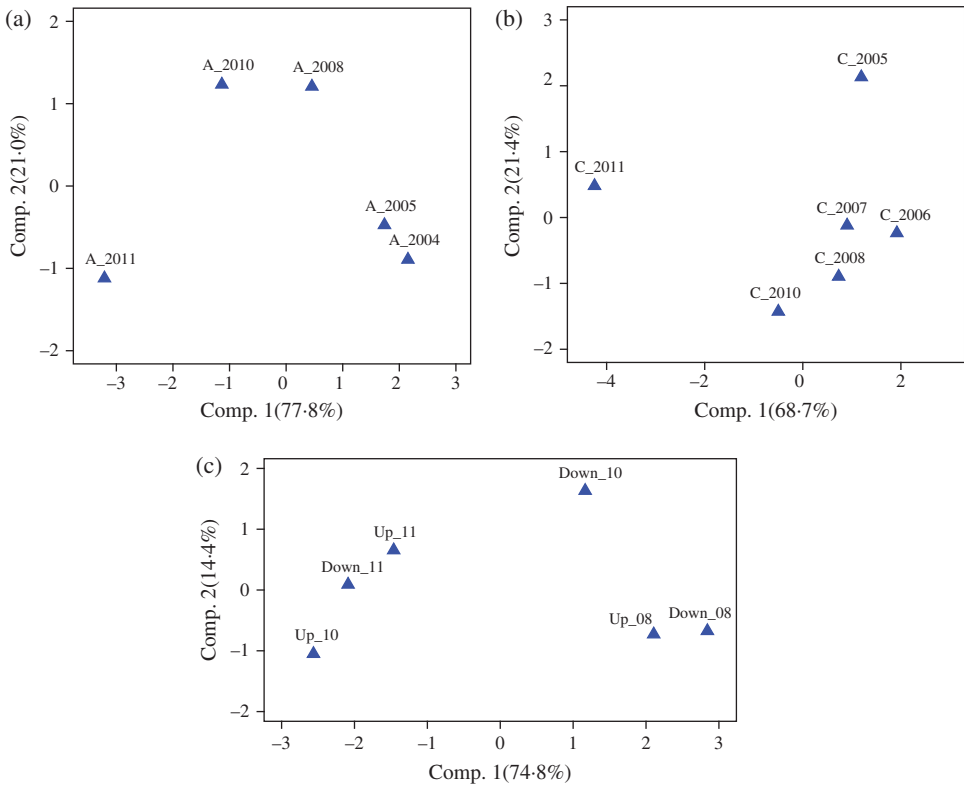


FIG. 2. Principal component analysis (PCA) based on pair-wise genetic distances  $[F_{ST} (1 - F_{ST})^{-1}]$  among (a) all samples, (b) all year-of-birth cohorts and (c) samples from upstream and downstream sections.

When considering all fish aged  $>1$  year for the analysis (*i.e.* adults plus the 2010 cohort), migration from upstream to downstream accounted for 17% of the fish present in the downstream section. Migration from the downstream to the upstream section was 2.86%.

Migration estimates when considering all fish (including individuals aged 0+ year) were 14.6% from upstream to downstream and 1.1% from downstream to upstream. In all analyses, high convergence was obtained across different runs.

## PARENTAGE ANALYSIS

Despite the low genetic variability retained in the Lipovscek Stream, an attempt was made to assess paternity for the 2011 cohort. In the downstream section, 31 of the 110 individuals aged 0+ year (28.2%) were assigned with a 100% probability to a single female adult, presenting three rare alleles not present in any other adult in the population. Six individuals aged 0+ year were assigned with a 100% probability to three adults from the downstream section, while seven individuals aged 0+ year were assigned with a 100% probability to two adults from the upstream section. The remaining 66 individuals aged 0+ year (60%) from the downstream section were assigned with the same probability to multiple parents and could not be unambiguously assigned to a single parent.



TABLE II. Estimation of effective population size ( $N_e$ ) using three different temporal (two-sample) estimators:  $F_K$  (Pollak, 1983),  $F_C$  (Nei & Tajima, 1981) and  $F_S$  (Jorde & Ryman, 2007), including confidence intervals

Cohorts	$F_K$	$F_C$	$F_S$
2005–2011			
$N_e$	36.4	40.2	20.9
Minimum	8.3	8.9	7.3
Maximum	332.8	590.3	41.7
2007–2011			
$N_e$	39.8	54.4	31.7
Minimum	8.2	9.8	11.0
Maximum	Infinite	Infinite	63.1
2008–2011			
$N_e$	22.7	26.5	11.5
Minimum	4.8	5.3	3.7
Maximum	276.0	2684.9	23.6
2010–2011			
$N_e$	14.6	13.0	12.5
Minimum	2.9	2.7	4.1
Maximum	Infinite	338.0	25.5

In the upstream section, 27 individuals aged 0+ year (31%) were unambiguously assigned: 13 to one adult, 13 to a second adult and one to a third adult. Moreover, three individuals aged 0+ year presented a genotype only compatible with a parent from the downstream section. The remaining 57 individuals aged 0+ year (65.5%) could not be unambiguously assigned to a single parent. Individual 13269, which contributed to about one third of the progeny of the downstream section, did not contribute to any of the progeny of the upstream section.

The relationship among the individuals from the 2011 cohort was also examined using COLONY. In the downstream section, a total of six full-sib pairs were inferred, all presenting the same multilocus genotype consisting of a combination of rare alleles at many loci. A total number of 178 half-sib pairs were inferred, although only 43 were

TABLE III. Mean  $\pm$  S.E. migration rates ( $m$ ) between the upstream and downstream sections of the river assessed using BayesAss considering the individuals present in the Lipovscek Stream in 2011: (a) individuals aged >1+ year and (b) all fish (including individuals aged 0+ year)

	$m$ (From upstream to downstream)	$m$ (From downstream to upstream)
(a) >1+ year fish ( $n = 25$ )		
Run_1	0.1508 $\pm$ 0.0705	0.0345 $\pm$ 0.0454
Run_2	0.1894 $\pm$ 0.0803	0.0227 $\pm$ 0.0240
(b) All fish ( $n = 222$ )		
Run_1	0.1476 $\pm$ 0.0526	0.0112 $\pm$ 0.0100
Run_2	0.1456 $\pm$ 0.0511	0.0113 $\pm$ 0.0103

$n$ , sample size.

significant at the 0.95 level. In the upstream section, a total of 12 full-sib pairs were inferred, although only one pair had a  $P$  value  $> 0.95$ . A total of 116 half-sib pairs were inferred, but only six of them showed a  $P$  value  $> 0.95$ .

## DISCUSSION

### SIGNATURES OF GENETIC EROSION

Monitoring of genetic diversity of *S. marmoratus* in the Lipovscek Stream during the period 2004–2011 shows signatures of genetic erosion with an important loss of genetic diversity, as evidenced by the decrease in observed and expected heterozygosities, polymorphism, minimum and total number of alleles and allelic richness. The observed decline in genetic diversity is a direct effect of the high mortalities associated with catastrophic flood events affecting the Slovenian Alps (Pujolar *et al.*, 2011*b*). While in the past, extreme flood events recurred approximately every 50 years, in the last 10 years, the frequency of floods has increased significantly with floods recurring on average every 4 years. The Lipovscek Stream has experienced the highest occurrence of extreme autumn floods in the area, including major floods in 2004, 2007 and 2009. Mortalities in the river were so high that as a consequence of the extreme floods the Lipovscek population was reduced to 38 fish aged  $>1+$  year following the 2007 flood and to only 10 fish aged  $>1+$  year after the 2009 flood. Consequently, no individuals aged 0+ year were observed in 2009, while the 2010 cohort was limited to just 16 individuals aged 0+ year. Genetic erosion associated with small cohort sizes is apparent when comparing the 2010 ( $n = 16$ ) and 2011 ( $n = 197$ ) cohorts. The 2010 cohort presented *c.* 30% fewer alleles relative to the 2011 cohort (12 *v.* 17), with alleles lost at four loci.

Current genetic variability at Lipovscek ( $H_O = 0.076$ ,  $H_E = 0.074$ ) is lower in comparison with the nearby streams of Zadlascica ( $H_O = 0.163$ ,  $H_E = 0.173$ ), Sevnica ( $H_O = 0.110$ ,  $H_E = 0.102$ ) and Studenc ( $H_O = 0.189$ ,  $H_E = 0.205$ ) analysed with the same markers used in this study (Pujolar *et al.*, 2011*b*). As mentioned above, this result is expected since Lipovscek has experienced a higher number of major floods than the other streams (*i.e.* Lipovscek had four major floods during the period 1999–2010 while Studenc only had one major flood). By comparison, Italian *S. marmoratus* populations showed higher heterozygosities ( $H_O = 0.15–0.39$ ) (Pujolar *et al.*, 2011*a*). Three loci were shared across studies showing only three alleles in the Slovenian populations but between seven and 17 alleles in the Italian populations. While Italian *S. marmoratus* populations are not experiencing floods, they are subjected to the negative effect of introgression with *S. trutta* (Pujolar *et al.*, 2011*a*). Similar heterozygosities ( $H_O = 0.16–0.35$ ) have been observed in the Prespa trout *Salmo peristericus* Karaman 1938 in Macedonia (Berrebi *et al.*, 2013), another endangered salmonid as a result of anthropogenic factors including reintroductions. The low heterozygosities found in the threatened *S. peristericus* and *S. marmoratus* (this study) contrast with the microsatellite heterozygosity values generally found in salmonids, ranging between 0.6 and 0.8 (Hansen *et al.*, 2002; Jensen *et al.*, 2005; Serbezov *et al.*, 2008).

Contemporary effective population size was estimated between 11 and 55 individuals, with similar values obtained using three different temporal estimators. This is congruent with the previous study of Pujolar *et al.* (2011*b*) that estimated a current  $N_e$

of 32 (C.I.: 1–259) individuals for Lipovscek using a Bayesian approach (MSVAR). As expected,  $N_e$  values are lower than census numbers, as not all adults contribute to spawning. Census size in Lipovscek was around 1000 individuals in 2005–2006 but dropped to <50 adults after the 2007 flood and remained low until 2011 (Fig. 1), which is congruent with the  $N_e$  estimations.

Owing to the high mortalities in the river following the floods, few adults might be contributing to the progenies, which would explain the low genetic variation found in the study. Parentage analysis of the 2011 cohort showed how c. 30% of the progeny of the downstream section was unequivocally assigned to one single female adult. This value could be actually higher as the genotype of this individual was compatible with all except five out of all non-assigned individuals (assigned to multiple parents). In fact, the software COLONY suggested that many of the individuals aged 0+ year from 2011 could be full or half siblings, which would explain the low heterozygosities and allelic richness observed in the 2011 cohort despite the large cohort size relative to the previous ones. For instance, allelic richness was higher in the 2008 cohort (1.86) than in the 2011 cohort (1.67), despite the 2008 cohort being only 10 individuals.

#### UPSTREAM–DOWNSTREAM GENE EXCHANGE

In Slovenian streams, the seven remnant genetically pure *S. marmoratus* populations survive in secluded headwaters above impassable waterfalls. This is the case of Lipovscek, which is divided into an upstream and a downstream section separated by a waterfall, which allows downstream movement while greatly limiting upstream movement. Genetic analysis and tagging data confirm that the two sections are not completely isolated and that there is some gene exchange between them. First, the data suggest a 15–19% migration of individuals from upstream to downstream, which is plausible as fish can easily be flushed down following the current. The existence of substantial downstream migration was supported by paternity analysis since genotypes of some individuals aged 0+ year found in the downstream section were only compatible with those of adults found in the upstream section of the river. Second, the data also suggest a small (1–3%) migration from downstream to upstream. In this sense, in the sampling, one individual first captured in June 2008 in the downstream section of the stream was re-captured in September 2008 in the upstream section, demonstrating that some upstream migration is certainly plausible. Later, a second identical case was observed.

Asymmetric gene flow appears to be common in salmonids (Fraser *et al.*, 2004). In *S. trutta*, the study of Hansen *et al.* (2007) revealed asymmetric gene flow from the two largest populations in the Hardanger Fjord (Norway) to the other five smaller populations studied, which has important implications as it constrains local adaptive responses in the smaller populations. In a recent study, Hansen *et al.* (2014) investigated the effects of medieval dams in two landlocked *S. trutta* populations in the Gudenå River (Denmark). Upstream migration was virtually zero, as would be expected given the difficulty in passing the dams upstream, while downstream migration was significant, as fish can pass the dams downstream by simply being flushed with the current. This pattern is similar to the one found in this study, with the difference that a signal of upstream gene flow was found in the latter. As mentioned above, gene flow in this direction is certainly a possibility, as fish can climb the waterfall in Lipovscek during flooding periods.

## PERSISTENCE DESPITE LOW GENETIC VARIABILITY

While there are apparent signatures of genetic erosion in the Lipovscek population with a general decline in genetic variability during the period 2004–2011, an important part of the genetic variation of the population has been retained. Allele loss has only affected rare alleles and all alleles with frequencies >5% that were present in 2004 were still observed in 2011, including many alleles with frequencies <10%. For instance, allele \*339 at locus *CA059136* showed a frequency of 6.7% at both 2004 and 2011 samples. Allele \*283 at locus *CA050376* changed from a frequency of 6.7% in 2004 to 3.2% in 2011. Other alleles have experienced larger changes in frequency, e.g. *Str85\*179* changed from 6.5% in 2004 to 27.1% in 2008 and 17.9% in 2011, but still remain in the population. Major changes in allele frequencies are mostly attributable to bottleneck and random drift effects due to reduced population sizes as a consequence of flood events. On the other hand, frequencies of most common alleles remained generally unchanged. For instance, allele \*337 at locus *CA059136* increased from 88.3% in 2004 to 89.9% in 2011 or allele \*154 at locus *Str591* increased from 70 to 75.5%. Other alleles showed larger fluctuations in frequency, again as a consequence of low population sizes following the flood events, i.e. *Str85\*171* increased from 56.5% in 2004 to 81.4% in 2011 and *Str543\** increased from 51.7% in 2004 to 77.8% in 2011.

Low genetic diversity has not prevented the survival of Slovenian *S. marmoratus* populations throughout time, which might be a common feature of small salmonid freshwater populations (Hendry & Stearns, 2004; Fraser *et al.*, 2011). Similarly, Valiente *et al.* (2007) questioned the importance of genetic variability, as three non-native salmonids [Atlantic salmon *Salmo salar* L. 1758, rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and *S. trutta*] were successfully introduced in Patagonian lakes despite showing low heterozygosities. Despite evolutionary potential being unquestionably related to genetic variation, a low genetic variability by itself does not appear to be an obstacle for successful adaptation and survival.

## POPULATION RECOVERY AND FUTURE CHALLENGES

The relaxation of density-dependent body growth and survival at low densities contributes to the resilience of stream-dwelling salmonids, which often rebound quickly to pre-event population levels (Vincenzi *et al.*, 2008a, 2012a). This can also be observed in the data for Lipovscek, in which following the 2009 flood, the 2010 cohort consisted of only 16 individuals aged 0+ year, while 1 year later the 2011 cohort consisted of 197 individuals aged 0+ year. A fast recovery of a stream salmonid population following a catastrophic flood was also observed in Gila trout *Oncorhynchus gilae* (Miller 1950) (Propst & Stefferud, 1997), with densities going back to normal 4 years after a flood that caused a >90% reduction in numbers. A fast recovery was also observed in a brook trout *Salvelinus fontinalis* (Mitchill 1814) population from Virginia affected by a massive flood and debris flow (Roghair *et al.*, 2002), with population densities exceeding pre-event levels after a period of 3 years. A similar recovery period was observed for *O. mykiss* in the Klamath Mountains (northern California) following catastrophic debris flows on stream ecosystems (Lamberti *et al.*, 1991).

The estimation in the present study of an effective population size of 11–55 individuals is one order of magnitude lower than the threshold of 500 individuals, proposed to be large enough to maintain genetic diversity for key life-history traits (Frankham, 1995). Moreover, floods in the Slovenian Alps have been intensified in the last 10 years and

occur now at higher frequencies and intensities than usual. Despite the prompt recovery of the *S. marmoratus* population of Lipovscek following several floods, there is concern that the predicted increase in frequency and intensity of floods in the next 50 years for the Adriatic basin of Slovenia (Lehner *et al.*, 2006) may challenge the resilience of *S. marmoratus*. Then, *S. marmoratus* might not have the sufficient potential to cope with such environmental changes, which could lead to the extinction of locally adapted populations. Future studies should focus on exploring the adaptive potential of these populations using genomic data, together with fitness experiments to assess the fitness consequences of inbreeding.

We thank Fondation MAVA and the Tolmin Angling Association for funding this study. All the people, especially D. Jesensek, Manager of the Association, and those who have participated each year to the fieldwork are warmly thanked.

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