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Fish migrate underground: the example of *Delminichthys adspersus* (Cyprinidae)

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Abstract

Complex aquatic systems of karst harbour a rich but little-investigated biodiversity. In Croatia and Bosnia–Herzegovina karst, temporal springs are inhabited by a group of minnow-like fishes that retreat to the associated ground water during dry seasons and spend several months underground. The most abundant species in this group is *Delminichthys adspersus* (Heckel 1843), which also has the most fragmented distribution range. To determine the population composition and dispersal patterns, and to detect potential underground migration, a large genetic data set comprising 544 specimens of *D. adspersus* covering most of its distribution area was analysed. Analysis of mitochondrial cytochrome *b* sequences (~1000 bp) and eight microsatellite loci showed that *D. adspersus* comprises at least three subpopulations with gene flow occurring among them. Coalescent-based analysis revealed a complex migration pattern, with several unidirectional dispersal paths, including between temporal springs that share no surface connection. The results of this study suggest the existence of recurrent underground migration of fish in a karst environment and demonstrate the complexity of its hydrological network. The findings are relevant to conservation strategies for endemic karst organisms and karst ecosystems as a whole.

Keywords: complex aquifer, cyprinids, Dinaric karst, isolation-with-migration model, population structure

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Introduction

Migration is a distinct feature of the life cycle of many fishes, ranging from spectacular long-distance spawning migration of several salmonid species to shorter, less noticeable movements in other species spanning just a few kilometres. The causes and reasons for migration can include reproduction, diet and predator avoidance, as well as colonization of new niches or newly formed water bodies (Lucas & Baras 2001). Even though numerous studies have investigated ecological, physiological, morphological and biogeographical aspects of epigenic fish migration (Detenbeck *et al.* 1992; Bernatchez & Wilson 1998; Høgåsen 1998; Allouche *et al.* 1999; Zardoya & Doadrio 1999; Strecker *et al.* 2004), we are unaware of any that have explicitly studied or reported underground migration. Published information on underground fish migration is limited to Atlantic salmon (*Salmo salar*), which has been observed to move through water diversion tunnels built for hydroelectric power purposes (Gowans *et al.* 2003), and to a proposed scenario of prehistoric dispersal of softmouth trout (*Salmo obtusirostris*; Snoj *et al.* 2008).

Karst regions comprise irregular limestone or dolomite landscapes containing sinkholes, sinking streams, caves and well-developed underground drainage systems, all characterized by a strong interaction between surface and ground water (Bonacci 1987, 1993; Field 2002). Surface water often arises at the edges of depressed flat plains or fields (polje), which are separated by mountains formed when limestone between insoluble rocky massifs dissolves away. Such water sources form permanent or temporal karst springs and

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streams, which can become strong torrents. At the beginning of the dry season, the water level drops and recedes underground in the porous karst fields, surviving sometimes only at the source (Kranjc 2004).

One of the best-studied karst areas, and one well known for its high level of biodiversity and endemism, is the Dinaric karst (Bonacci 1987) that stretches from Slovenia to Albania in the Balkan Peninsula. Endemic species include Martino's vole *Dinaromys bogdanovi* (Kryštufek *et al.* 2007), *S. obtusirostris* (Snoj *et al.* 2008), the only European stygobiontic amphibian, *Proteus anguinus* (Sket *et al.* 2004), *Bryocamptus zschokkei* (Copepoda), *Velkovrhia enigmatica* (Hydrozoa) and *Troglocaris* sp. (Decapoda) (Sket *et al.* 2004; Verovnik *et al.* 2004) and a group of poorly studied small cyprinids (*Telestes*, *Phoxinellus* and *Delminichthys*) with the vernacular name 'gaovice' (Mrakovčić *et al.* 2006).

Gaovice, particularly those of the genus *Delminichthys*, are restricted to the mouths of springs and associated ground water. During the dry season, the fish actively follow water that is withdrawing underground, where they can live continuously for several months (Zupančič & Bogutskaya 2000; Bogutskaya & Zupančič 2003; Mrakovčić *et al.* 2006). The most abundant species of this group is *Delminichthys adspersus* (Heckel 1843), which has a fragmented distribution range in southeastern Croatia and southern Bosnia–Herzegovina. The species inhabits Red Lake (Crveno jezero; type locality) and many direct and indirect springs of the Vrljika, Tihaljina and Matica river systems (Zupančič 2008; Fig. 1). Apart

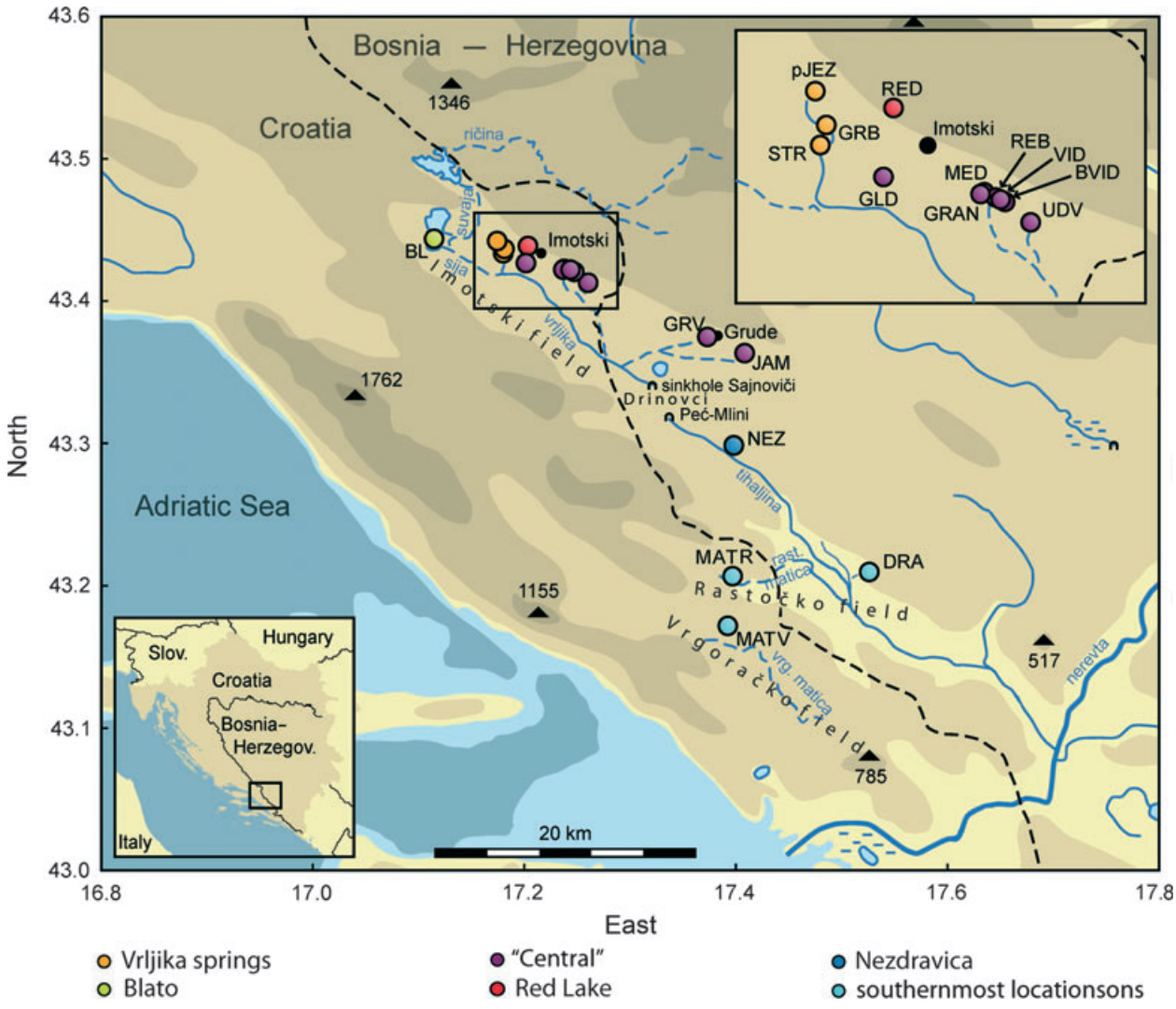

Fig. 1 The study area is part of the Dinaric karst of the Balkan Peninsula, ranging from the southeastern Croatia to southern Bosnia–Herzegovina. Eighteen sampling locations include type locality Red Lake, and direct and indirect springs of the Vrljika, Tihaljina and Matica river systems, which cover most of the distribution area of *D. adspersus*. See text for a detailed description and Table 1 for abbreviations of sampling locations.

Table 1 Sampling locations for *Delminichthys adspersus* with respective latitude, longitude, elevation and abbreviations, with the number of specimens per sampling location, number analysed for mtDNA analysis and group assignment based on genetic structure and geographic proximity reported (GROUPS)

Location	Abbr.	No. of specimens (included in mtDNA analysis)	Latitude and Longitude	Elevation a.s.l. (m)	GROUPS	River system
Vrgoračka Matica River	MATV	10 (0)	/	Approx. 30	Southernmost locations	Tihaljina river system
Rastočka Matica River	MATR	27 (20)	N 43 13 07.03 E 017 23 14.29	65		
Draga	DRA	16 (15)	N 43 13 15.7 E 017 30 41.5	76		
Nezdravica	NEZ	46 (21)	N 43 18 58.45 E 017 23 20.29	161	Nezdravica	
Jezerine, Proložac	pJEZ	53 (19)	N 43 27 25.77 E 017 10 20.93	269	Vrljika springs	Vrljika river system
Grbavac	GRB	34 (18)	N 43 27 06.22 E 017 10 36.67	275		
Stari vodovod	STR	19 (16)	N 43 26 52.41 E 017 10 34.1	274		
Blato	BL	29 (23)	N 43 27 30.59 E 017 06 44.01	281	Blato	
Grančice (Imotski springs)	GRAN	24 (21)	N 43 26 15.48 E 017 13 3.82	268	'central'	
Medvidovića Draga (Imotski springs)	MED	17 (17)	N 43 26 18.41 E 017 13 41.64	268		
Rebić (Imotski springs)	REB	23 (21)	N 43 26 05.2 E 017 14 26.4	258		
Glavina Donja (Imotski springs)	GLD	37 (20)	N 43 26 36.76 E 017 11 42.93	274		
Udovice (Imotski springs)	UDV	28 (21)	N 43 25 39.2 E 017 15 15.36	261		
Vinjani Donji, bunar (Imotski springs)	BVID	25 (19)	N 43 26 05.32 E 017 14 26.4	264		
Vinjani Donji (Imotski springs)	VID	40 (19)	N 43 26 11.35 E 017 14 20.33	264		
Grudsko Vrijelo (Grude springs)	GRV	24 (15)	N 43 23 22.45 E 017 22 04.78	263		
Jamine (Grude springs)	JAM	40 (15)	N 43 22 29.35 E 017 23 23.77	273		
Red Lake (Crveno jezero)	RED	33 (37)	N 43 27 14.84 E 017 11 54.93	452	Red Lake	

from information on its phylogenetic position (Freyhof *et al*. 2006; Palandačić *et al*. 2010; Perea *et al*. 2010) and distribution and morphology (Zupančič & Bogutskaya 2000; Bogutskaya & Zupančič 2003; Franičević & Tičina 2003), little else is known about this species, with published information on its biology and ecology being scarce and vague (Bilopavlović 1970; Mrakovčić *et al*. 2006). Previous research indicates that *D. adspersus* is not generally observed in areas beyond the springs (authors' personal observation).

The entangled water network that *D. adspersus* inhabits represents an ideal system to study the species' population dynamics. In this study, we analysed the genetic structure of populations and compared the distribution of their genetic diversity with their geographic distributions in order to determine the population migration patterns of the species. Finally, we compared the evidence for recurrent underground migration with that for other explanations such as recent divergence or secondary contact.

Materials and methods

Sampling sites

In this study, 18 sampling sites were chosen to cover most of the distribution area of *D. adspersus* (Zupančič

Fig. 2 Red Lake (Crveno jezero) is a 500-m-deep sinkhole with the water level 250 m below the lip.

2008) (Fig. 1, Table 1). Some sites are mutually surface-isolated or belong to separate water systems without surface connection. These include Red Lake (Crveno jezero, RED), a 500-m-deep sinkhole with a water level 200–250 m below the lip (Fig. 2, Garašić 2001; Bonacci 2006), the Vrljika and Tihaljina rivers systems, which are connected only underground, and the intermittent Vrgoračka Matica River (MATV), which springs and sinks in Vrgoračko field (Kanaet 1959; Džeba 2010). We also included sites that are periodically linked via a network of temporal streams or canals, but because of several obstacles (e.g. distance, high temperature, drying up, differences in elevation, strong current at spring outlet), surface fish dispersal among them is highly unlikely. These sites include Blato (BL), Vrljika River springs (pJEZ, GRB, STR) and Grude springs (GRV, JAM) (Vrljika river system; Džeba 2010) and the rivers Nezdravica (NEZ), Draga (DRA) and Rastočka Matica River (MATR) (Tihaljina river system; Kanaet 1959). Imotski springs (GRAN, MED, REB, BVID and VID with the exception of GLD and UDV) interconnect via overflow drains and flooding (authors' personal observation, Fig. 1). All sampling sites are described in more detail in Table 1 and the Supporting information.

On the basis of the genetic similarity of fish from different sampling sites, described in the Results section, six panmictic genetic groups were recognized (see Table 1), representing units for gene flow analysis.

Sample collection and DNA isolation

A total of 544 individual samples were collected using electrofishing or fish traps (Fig. 1, Table 1). Most samples were collected between 2007 and 2010 with the exception of those from MATV, which dated from 1999. In this last sample set, mitochondrial DNA (mtDNA) amplification failed. Nevertheless, because of the highly demanding sampling conditions at this location, we included these samples for microsatellite analysis for which we obtained good results. A sample set from Red Lake was composed of two subsets originating from 2007 (n = 5) and 2010 (n = 35).

Genomic DNA was isolated from fin tissue preserved in 96% ethanol, using the salt extraction procedure of Miller *et al.* (1988).

Microsatellite DNA

Eight microsatellite loci, isolated and characterized from related cyprinid species and optimized to amplify in *D. adspersus* in two multiplex PCRs (see Supporting information), were chosen for genotyping of all the collected individuals. Genotyping was performed on an ABI-3130 automated capillary sequencer using GeneScan Analysis Software 3.7. Microsatellite data were processed using GeneMapper 4.0 (ABI) and edited by eye. Automated binning of allele sizes was performed with tandem 1.07 (Matschiner & Salzburger 2009). Possible null alleles, stuttering and large allele drop-out were tested using Micro-checker (Van Oosterhout *et al.* 2004). The average numbers of alleles, expected and observed heterozygosity were estimated with Arlequin.

Population structuring within the data set was assessed using the Bayesian-clustering iteration method (programs structure 2.3.1 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009) and baps (Corander *et al.* 2003; Corander & Marttinen 2006; Corander *et al.* 2006, 2008), assuming no a priori assignment of individuals. In structure, inferences on the number of clusters are based on an *ad hoc* approximation with a generally unknown performance (Pritchard *et al.* 2000), while baps calculates posterior distribution of partitions among the sampling units into nonempty classes, each with nonidentical allele frequency parameters (Corander *et al.* 2008). The chosen settings for structure and baps that ensure MCMC convergence are described in the Supporting information. The method of hierarchical structure partitioning suggested by Vähä *et al.* (2007) was also applied. With this approach, the most differentiated cluster was excluded from the analysis, allowing for more precise clustering of the remaining individuals. Also, each excluded cluster was investigated for possible hidden substructures (see Supporting information). To meet the requirements of Bayesian methods for unlinked genetic markers, the independence of the chosen microsatellite loci was verified with pairwise tests for linkage disequilibrium (Slatkin & Excoffier 1996) implemented in Arlequin (see Supporting information).

The amount of interpopulation genetic differentiation of *D. adspersus* was quantified by computing F_{ST} values for all pairs of sampling sites. Pairwise tests of genetic differentiation were conducted using Arlequin (F_{ST}; 1000 permutations, 5% missing data and nominal levels 0.05, 0.01 and 0.001). F_{IS} (Weir & Cockerheim 1984) was calculated using FSTAT 2.9.3.2 (Goudet 2001) for the groups pooled according to pairwise F_{ST} analysis. In all tests, *P*-values were adjusted using Bonferroni correction (Rice 1989).

Hierarchical AMOVA was carried out using Arlequin, where variance components were estimated for three hierarchical levels: among groups, between sampling sites within groups and within sampling sites.

In order to determine the direction and amount of gene flow between six groups (see Table 1), pairwise analyses with the isolation-with-migration model (IM, as implemented in the software IMa2; Hey & Nielsen 2007) were performed. For each analysis, up to 36 individuals were drawn at random from the two included groups. Where possible (given sufficient number of samples), multiple independent runs with a different set of individuals were performed to assess the run convergence and data reliability (see Supporting information for details).

Previous versions of IMa2 provided estimates for the timing of gene flow events that have commonly been used to infer the mode of speciation (Niemiller *et al.* 2008; Nadachowska & Babik 2009). However, recent simulation studies (Becquet & Przeworski 2009; Strasburg & Rieseberg 2011) have shown that these estimates are unreliable, and the software authors have removed the estimation of migration times from the latest version of the program. We could not therefore use these estimates to investigate whether gene flow is recent or historical. Instead, a genetic assignment method for direct real-time estimation of first-generation migrants (Paetkau *et al.* 2004), implemented in Geneclass2 (Piry *et al.* 2004), was applied (for chosen settings, see Supporting information) to assess whether gene flow is recent.

To determine whether stepwise-like mutations have contributed to genetic differentiation (Hardy *et al.* 2003), allele size (R_{ST}) and the allele identity–based measure (F_{ST}) were compared by testing whether the observed R_{ST} was larger than the value obtained after permuting allele sizes among alleles within populations as implemented in SPAGeDI 1.3 (Hardy & Vekemans 2002; 20 000 permutations). To test how changes in migration rates vs. mutation rates influence population structure, some simple simulations with EASYPOP 1.7.4 (Balloux 2001) were performed (see Supporting information).

Possible genetic bottlenecks were investigated using Bottleneck 1.2.02 (Piry *et al.* 1999). The analysis was performed with a two-phased mutation model with multistep mutations accounting for ten per cent of all mutations and ten per cent of the variance. Because fewer than 20 loci were genotyped, Wilcoxon's test was used as suggested by Piry *et al.* (1999).

Mitochondrial DNA

Nucleotide sequences of the mitochondrial cytochrome *b* gene were analysed to obtain the supplementary information about geographic clustering and demographic expansion of *D. adspersus*. The cytochrome *b* gene was amplified from a subset of randomly selected specimens from each sampling location with the exception of MATV (n = 15–37; Table 1). Detailed information on PCR and DNA sequencing is reported in the Supporting information. Sequences were edited by eye and aligned using the software MEGA 5.0 (Tamura *et al.* 2011).

In order to assess the population structure in relation to geographic distribution, a haplotype genealogy was built on the basis of the phylogenetic tree constructed using the TrN model of sequence evolution (Tamura & Nei 1993) and the maximum-likelihood method implemented in PAUP* 4.0 (Swofford 2003; for details, see Supporting information). Nucleotide diversity (Nei 1987) was calculated using the software package Arlequin 3.11 (Excoffier 2007).

The demographic history (information about historical episodes of expansion or decline) at the species level was tested by applying mismatch analysis (Li 1977), for which all variant mtDNA sequences were included. The distribution of pairwise mutational distances was fitted to a model of instantaneous population expansion by a generalized nonlinear least squares procedure (Schneider & Excoffier 1999), as implemented in Arlequin. Statistical significance of model fit was assessed by squared deviation and raggedness index (Harpending 1994) and 10 000 bootstrap replicates.

Results

Microsatellites

A total of 544 individuals of *D. adspersus* from the 18 different sampling locations were scored for eight microsatellite loci. Individuals that failed to be genotyped at three or more loci were excluded from the analysis, which finally resulted in 522 genotyped specimens.

As revealed by Micro-checker analysis, none of the loci at any sampling site exhibited null alleles, with the exception of the Ca3 locus in the composed sample set of Red Lake. After analysing each subset separately, null alleles were no longer observed. There were no indications of stuttering or allele drop-out.

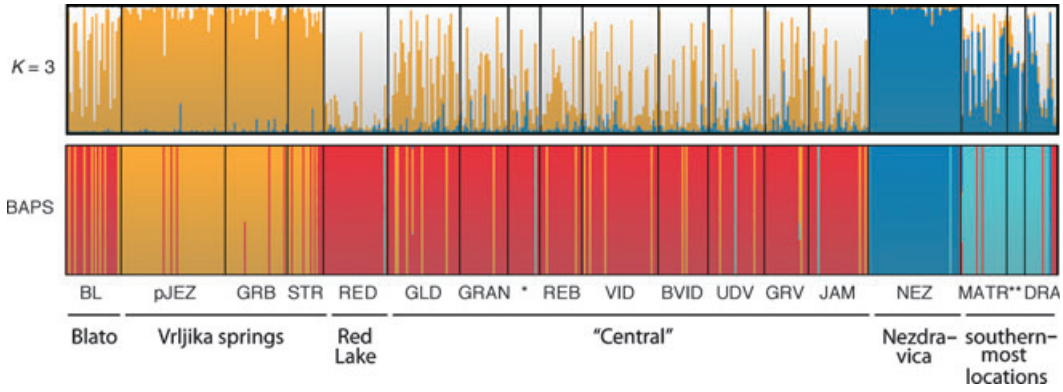

Fig. 3 Bayesian-clustering analyses: Structure (K = 3) and BAPS; *MED; **MATV.

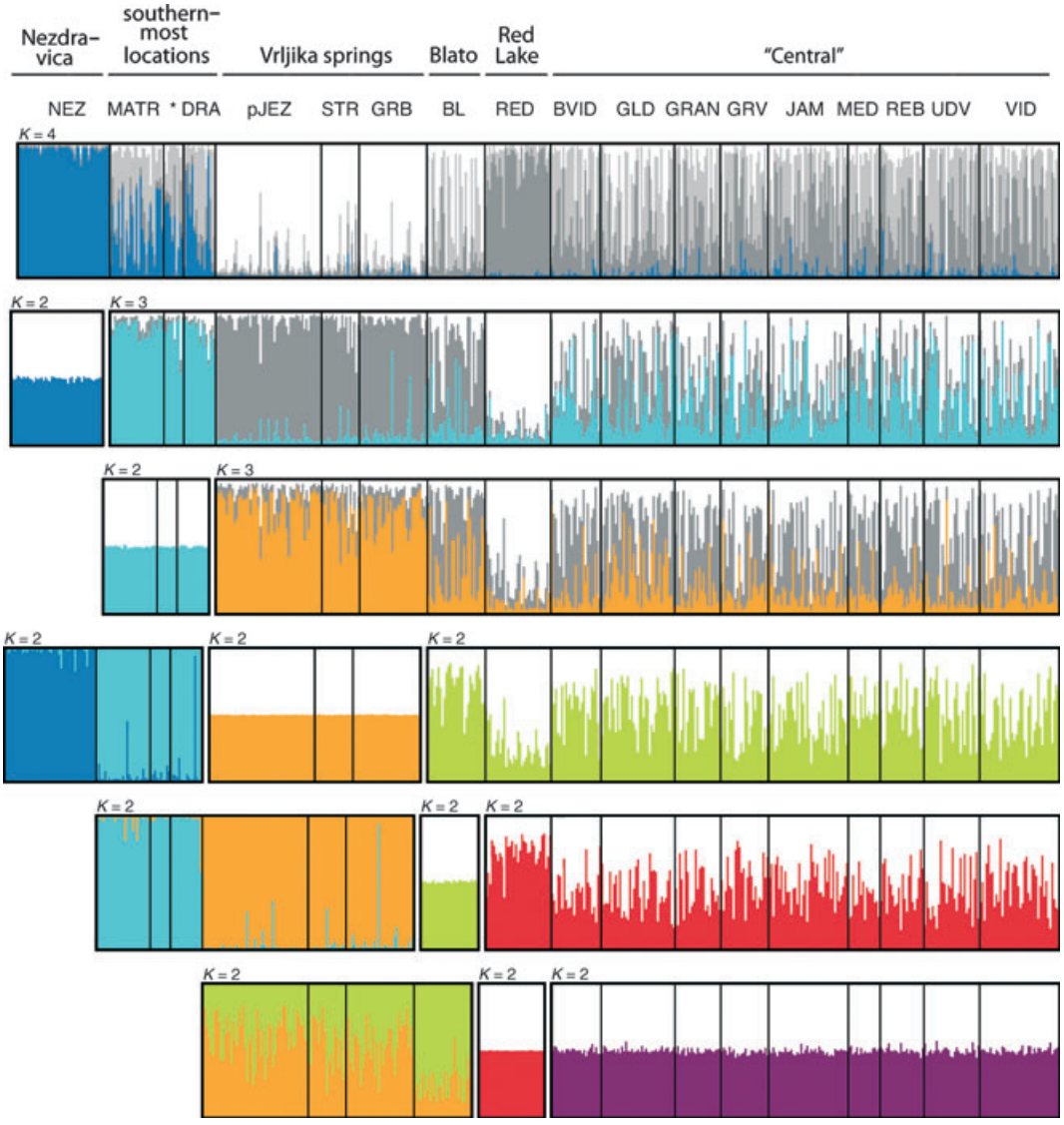

Fig. 4 In hierarchical STRUCTURE analysis, stepwise exclusion of the most differentiated group was conducted, allowing for a more precise clustering of the remaining individuals. Each excluded group was analysed to check for cryptic subpopulations. The analysis identified six distinct groups.

Table 2 Population parameters

GROUP	Location abbr.	*N*	F_{IS}	Pooled F_{IS}	N_A	H_{EXP}	H_O
Southernmost locations	DRA	16	0.111	0.012	9.750	0.710	0.796
	MATR	27	−0.031		11.000	0.822	0.798
	MATV	10	−0.003		7.250	0.779	0.772
Red Lake	RED	33	0.073	0.073	10.750	0.676	0.728
'central'	GLD	37	0.026	0.016	13.500	0.785	0.806
	BVID	25	0.010		13.000	0.773	0.780
	REB	23	0.020		13.000	0.785	0.800
	GRAN	24	0.030		12.375	0.776	0.795
	MED	17	0.060		11.625	0.760	0.807
	UDV	28	0.029		12.875	0.770	0.792
	VID	40	−0.041		15.625	0.832	0.800
	GRV	24	0.007		11.500	0.787	0.792
	JAM	40	0.021		14.125	0.784	0.801
Nezdravica	NEZ	46	0.012	0.012	5.500	0.615	0.622
Vrljika springs	pJEZ	53	0.004	−0.024	11.250	0.719	0.721
	GRB	34	−0.086		10.375	0.817	0.754
	STR	19	0.001		9.250	0.748	0.749
Blato	BL	29	−0.013	−0.013	12.625	0.779	0.769

N, number of individuals; F_{IS} (FSTAT) for each population and for pooled groups; N_A, average number of alleles per locus; H_{EXP}, expected average heterozygosity; H_O, observed heterozygosity (Arlequin).

Abundant microsatellite diversity was observed in *D. adspersus*: the average number of alleles across all loci was 11.4 (SD = ±2.4), and expected heterozygosity across all loci was 0.761 (SD = ±0.053). Tests for linkage disequilibrium found no correlation between the microsatellite loci chosen in the present study, thus meeting the requirements for the Bayesian methods applied here.

After assigning individuals to $K = 1$–7 groups using STRUCTURE (Fig. 3), the most probable number of clusters in the data set was estimated to be $K = 3$, composed of individuals collected from Nezdravica, from Vrljika springs and from Red Lake. Other individuals were, to a varying extent, assigned to all three clusters. Hierarchical STRUCTURE analysis did not reveal hidden clusters within these three clusters but rather suggested three more groups (Fig. 4): (i) MATR–MATV–DRA (hereon referred to as the 'southernmost locations'), (ii) Imotski springs and Grude springs (hereon referred to as 'central') and (iii) Blato.

Cluster analysis with BAPS supported genetic homogeneity of Nezdravica, Vrljika springs, Red Lake and the southernmost locations, while the remaining clusters, suggested by hierarchical STRUCTURE analysis, represented genetically mixed groups. The individuals from Blato were assigned to Vrljika springs and Red Lake, while the individuals from 'central' were mostly assigned to Red Lake, and a few to the southernmost locations and Vrljika springs (Fig. 3).

Using pairwise F_{ST} values, significant genetic differentiation was observed between the six groups previously recognized with hierarchical STRUCTURE analysis (for details, see Supporting information). F_{IS} values (Table 2) observed in each of the six groups were not significant.

Hierarchical AMOVA revealed that most of the variation (93.3%, $P \leq 0.00001$) occurred within sampling sites and 6.5% among these six groups ($P \leq 0.00001$). However, molecular variation among sampling sites within the groups was not significant.

While all the clustering methods used in this study gave strong support for at least three groups (i.e. Vrljika springs, Red Lake and Nezdravica), clustering performed with BAPS, hierarchical STRUCTURE and *F*-statistics revealed additional panmictic groups (Blato, 'central' and southernmost locations) being supported also with AMOVA. As the IM model assumes that there should not be other unsampled populations exchanging genes with the sampled populations, and that populations are more closely related to each other than to any other sampled population (Hey, 2007), these six panmictic groups (Table 1) were employed for the analysis.

These six groups were consistent also with surface and underground water connections of the study area and potential barriers between them (Bonacci 2006; Kanaet 1959).

IMa2 analysis between the six groups revealed a migration pattern with many unidirectional migration paths (Fig. 5). The migration rate from Red Lake to 'central' was seven times larger than in the opposite direction. Unidirectional migration was further suggested from Vrljika springs to Blato, and from Blato to 'central', as well as from Nezdravica to 'central'. Also,

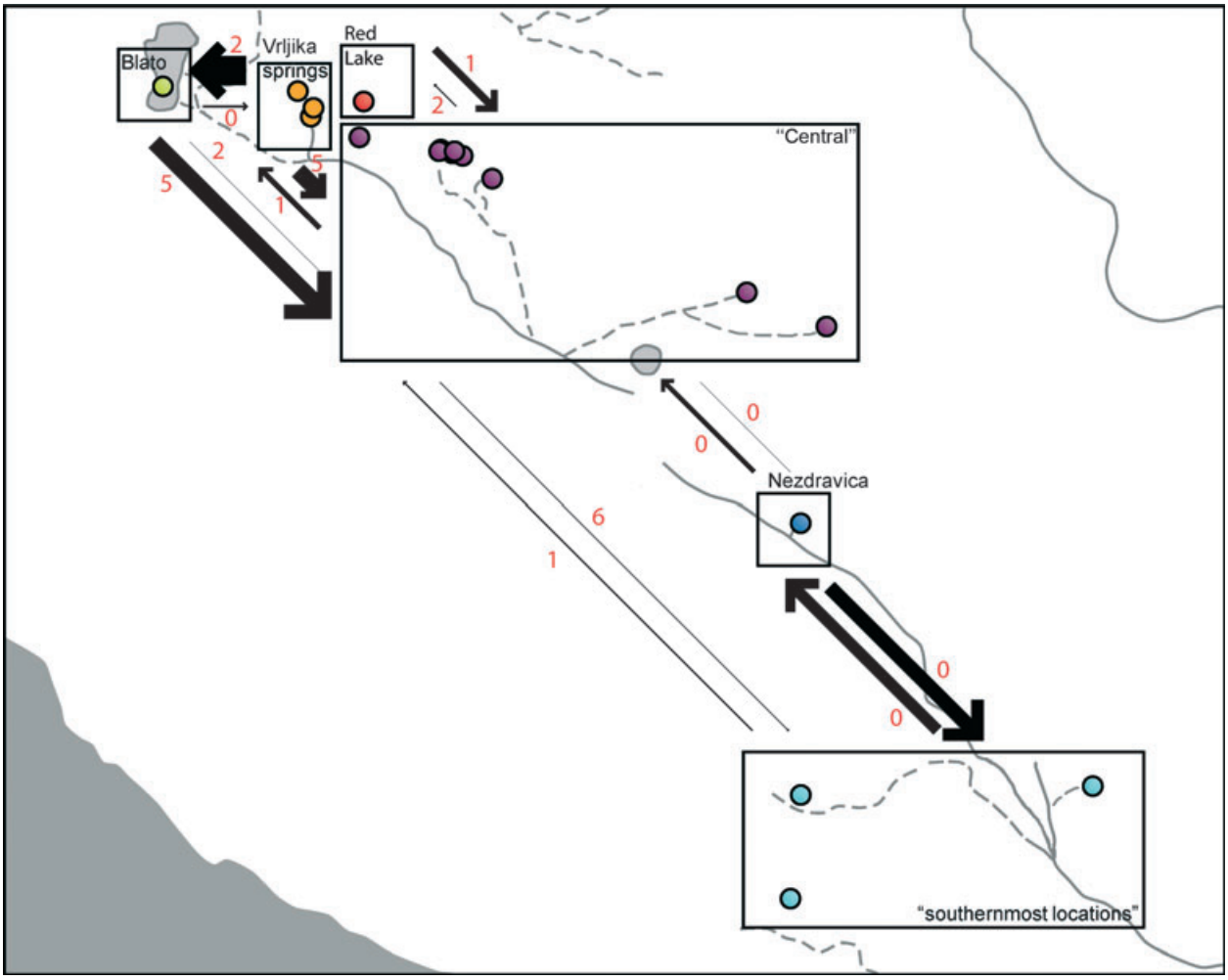

Fig. 5 Migration patterns according to IM model estimates. Arrow width directly corresponds to IMa2's HiPt estimate of migration rate (ranging from 0 between RED and Vrljika springs to 4 between Vrljika springs and BL); see Table 3 for details; red numbers correspond to potential first-generation migrants (Geneclass2).

the inferred migration rate from Nezdravica to the southernmost locations was larger than in the opposite direction. The only pair of groups with migration rate estimates between them of zero in both directions was Vrljika springs and Red Lake. The highest migration rate was found between Vrljika springs and Blato.

Although Imotski and Grude springs are geographically remote, they were recognized as the panmictic 'central' group. IMa2 analysis was undertaken to see how the model reacted when it was violated and migration tested within a panmictic group. The test estimated relatively high levels of migration (see Table 3), although the estimated split time was zero, as expected for molecular information drawn from a single panmictic population (J. Hey, personal communication). On the other hand, the estimated split time between any tested pair of the six groups was larger than zero, pointing to mutual divergence among them.

More detailed information about IM model parameter estimates (time since divergence, effective population sizes of ancestral and descendent populations, migration rates and population migration rates) and their convergence is reported in Table 3. Of those replicates that were repeated with other samples from the same populations, only the results of the replicate with better run convergence are reported, because the parameter estimates were very similar.

A total of 84 F_0 putative migrants were identified through the use of Geneclass2 ($\alpha = 0.05$). Migration was observed mostly within previously detected groups (Blato, Vrljika springs, Red Lake, 'central', Nezdravica, southernmost locations), although 32% (26 migrants) took place between them. The number of putative first-generation migrants between the groups is indicated in Fig. 5. The detailed description can be found in the Supporting information together with the Geneclass2 output file.

Table 3 IM model estimates: time since divergence (t_0), effective population sizes of ancestral and descendent populations (q_A, q_1, q_2), migration rates ($m_{0>1}$ and $m_{1>0}$) and population migration rates ($2N0_{m0>1}$ and $2N1_{m1>0}$) with the lower and upper boundaries of the 95% highest posterior density (HPD) interval. All parameters except population migration rates are scaled by mutation rate

Group pair	t_0 HiSmth (95%Lo–95%Hi)	q_A HiPt (95%Lo–95%Hi)	q_1 HiPt (95%Lo–95%Hi)	q_2 HiPt (95%Lo–95%Hi)	$m_{0>1}$ HiPt (95%Lo–95%Hi)	$m_{1>0}$ HiPt (95%Lo–95%Hi)	$2N0_{m0>1}$ HiPt (95%Lo–95%Hi)	$2N1_{m1>0}$ HiPt (95%Lo–95%Hi)
CENT–NEZ	0.223 (0.105–1.751)	73.75 (39.75–485.8)	0.75 (0.25–1.25)	20.25 (12.75–34.75)	0.62 (0.1–1.74)	0.02 (0.02–3.9)	18.81 (2.179–38.96)	0.007 (0.0111–0.8393)
CENT–MADR	1.599 (0.625–1.967)	16.75 (10.75–36.25)	10.25 (6.25–18.75)	36.25 (21.25–83.75)	0.18 (0.02–1.22)	0.1 (0.02–0.82)	3.098 (0.2599–9.975)	0.7556 (0.06985–3.651)
CENT–RED	0.285 (0.149–1.891)	27.75 (16.75–458.2)	2.75 (1.75–5.75)	19.75 (12.25–50.75)	1.06 (0.14–3.06)	0.14 (0.02–3.5)	15.39 (3.178–38.24)	0.6138 (0.07485–3.678)
CENT–BL	0.175 (0.069–1.905)	30.75 (19.25–480.2)	4.75 (2.75–10.75)	21.25 (12.75–53.75)	2.3 (0.18–5.98)	0.02 (0.02–4.38)	20.97 (2.139–38.96)	0.01999 (0.09995–8.376)
CENT–GPJS	0.239 (0.149–1.911)	97.75 (40.25–487.2)	2.75 (1.75–4.75)	21.75 (13.25–55.75)	2.34 (0.82–4.62)	1.22 (0.14–3.98)	39.96 (7.536–39.52)	1.934 (0.269–4.035)
RED–GPJS	0.315 (0.173–1.515)	3.25 (1.75–6.25)	3.75 (2.25–6.75)	27.25 (15.75–66.25)	0.02 (0.02–1.7)	0.02 (0.02–1.58)	0.00397 (0.02779–2.322)	0.00407 (0.03663–2.56)
BL–GPJS	0.123 (0.027–1.483)	5.75 (3.25–419.2)	3.25 (1.25–6.25)	20.75 (12.25–60.25)	4.06 (0.38–11.46)	0.3 (0.1–6.54)	12.01 (1.779–36.56)	2.099 (0.1399–7.816)
MADR –NEZ	0.689 (0.259–1.899)	236.2 (54.75–488.8)	0.75 (0.75–1.25)	30.25 (18.75–76.75)	1.98 (1.34–3.86)	1.62 (0.46–4.7)	39.96 (11.09–39.64)	0.6746 (0.245–1.267)
RCENT–JAGR	0.001 (0.001–1.565)	499.8 (36.25–488.2)	7.75 (3.25–454.2)	26.75 (18.25–56.25)	0.02 (0.1–13.14)	0.3 (0.06–6.54)	0.01999 (0.6597–38.6)	1.299 (0.3398–30.88)

RCENT, Imotski springs (7 locations); JAGR, Grude springs (2 locations); CENT, Imotski springs + Grude springs; NEZ, Nezdravica; MADR, Matica + Draga; GPJS, Grbavac + Proložac Jezerine + Stari vodovod; RED, Red Lake; BL, Blato.

The observed R_{ST} value of the whole sample set was 0.087, while the R_{ST} value observed after permutation was 0.037 ($P = 0.0018$), and the F_{ST} value was 0.051. Significantly larger R_{ST} in comparison with F_{ST} suggested that stepwise-like mutations contributed to genetic differentiation. Simulations performed in EASYPOP (for details, see Supporting information) suggested that equilibrium among isolated groups with occasional migrants is established after 10 000 generations, while in the simulated data set R_{ST} becomes significantly larger than F_{ST} as late as after 100 000 generations.

Bottleneck analysis of the data set as a whole and of the six genetic groups revealed no significant heterozygosity excess and therefore did not support the existence of recent bottlenecks.

Mitochondrial DNA

Sequencing of the *D. adspersus* cytochrome *b* gene resulted in alignment of 999 bp from 337 individuals representative of 17 sampling locations (GenBank Accession Nos. JN101952–JN102110 and JQ315936–JQ316113). The alignment contained no gaps or missing data and was collapsed to 74 haplotypes.

The haplotype genealogy exhibited a star-like pattern delineating three most common and most geographically widespread haplotypes: 1 (H1; 46% of all samples), 2 (H2; 19%) and 3 (H3; 7%) with H2 and H3 separated from H1 by only one mutation (Fig. 6). Most other haplotypes were represented by single specimens, were private to particular sampling sites and were divergent from H1 and H2 generally by one substitution (Fig. 6).

With regard to the six genetic groups, the most noticeable characteristic of the haplotype network was the almost complete absence of representatives from Vrljika springs in the H1 cluster, in which only one sample from this group was found, and a strong prevalence of H1 haplotype (81%) in Nezdravica. On the other hand, in the H2 cluster, which was common in Blato, Vrljika springs and 'central', only one sample from the southernmost locations and none from Nezdravica were present. The third largest cluster, H3, comprised mostly samples from Vrljika springs, while 'central' contributed four samples. Most Red Lake samples (68%) were attributed to haplotype H1, although some were also attributed to haplotype H2 (8%), while others were private (24%). A detailed list of haplotypes at each sampling site is reported in the Supporting information. Average nucleotide diversity across all loci was 0.001243 (±0.000873).

Mismatch analysis of the whole sample set detected a unimodal distribution of pairwise differences among haplotypes. However, the model of sudden population

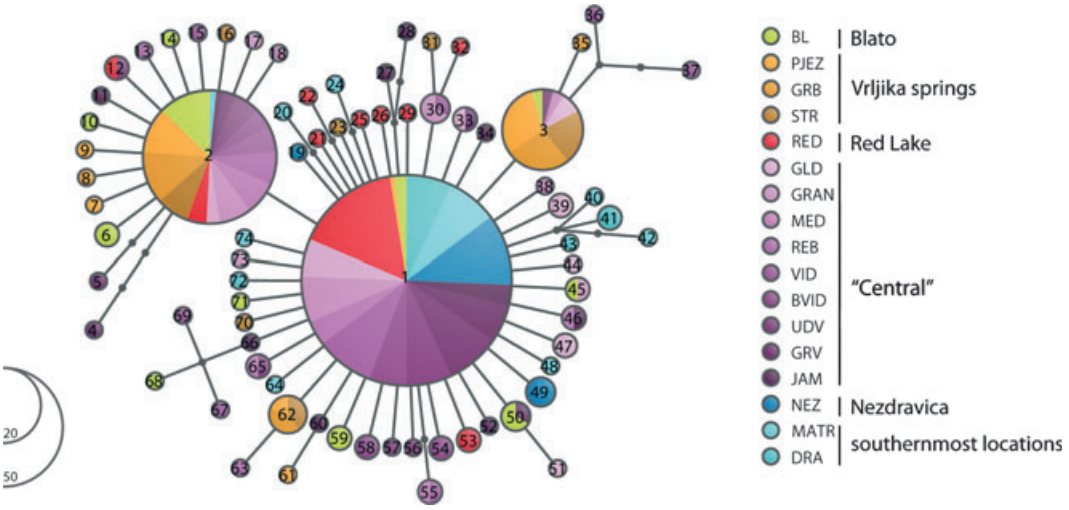

Fig. 6 Unrooted haplotype genealogy based on 337 cytochrome *b* gene sequences (999 bp). Labels refer to observed haplotypes (in the text referred to as H1, H2, H3, etc.); colours denote sampling sites, and radii reflect the number of individuals per haplotype.

expansion was rejected (sum of squared deviations; $P = 0.041$) and significant ($P = 0.002$) raggedness index showed variation around the curve, thus suggesting long-term demographic stability of the species.

Discussion

In the present study, the genetic composition of endemic cyprinid *D. adspersus* revealed low differentiation among geographically isolated populations. Although the genetic results in combination with hydrological data favour the hypothesis of recurrent underground migration, such low interpopulation differentiation could also be a consequence of long-term process of genetic differentiation and is to be expected in recently isolated systems. Low mutation rates, as suggested for the genus *Delminichthys* (Freyhof *et al.* 2006), may slow this process even further. Thus, two scenarios explaining the observed population genetic structure in *D. adspersus* are possible: (i) recent isolation of populations with no subsequent gene flow among them and (ii) recurrent, but limited, gene flow among diverged populations.

The results of mtDNA analysis, such as the star-like distribution of haplotypes and very low number of mutations among the three central haplotypes, imply a recent origin of the system (the first scenario). In the example of *Schistura oedipus*, a balitorid cave fish from Thailand, nucleotide diversity was estimated as 1×10^{-3} within populations and 5×10^{-3} between separated populations (Borowsky & Mertz 2001); the overall level of nucleotide diversity in *D. adspersus* is low (1.2×10^{-3}) and is in line with intrapopulation nucleotide diversity reported for balitorids, again suggesting recent origin of the system.

Opposed to the first scenario, the low interpopulation differentiation inferred from microsatellite data can be explained by recurrent gene flow (the second scenario), which can prevent the complete isolation of geographically isolated populations even with only a few migrants per generation (Hartl & Clark 1989). The scenario of gene flow among diverged populations is favoured also by $R_{ST} > F_{ST}$, which indicates a significant contribution of stepwise-like mutations in the observed interpopulation differentiation when compared to the effect of genetic drift, suggesting that the divergence is not recent (>ca. 10 000 years). On the other hand, if gene flow exists, low interpopulation divergence is expected and R_{ST} should not exceed F_{ST}. But $R_{ST} > F_{ST}$ does not exclude migration (Hardy *et al.* 2003) and can be explained by differentiated populations with a low level of gene flow between them (e.g. Gavrilets 2003), suggesting that in *D. adspersus* the migration rate among the detected groups is rather limited. Basic simulations of four populations with a single-step migration model revealed that R_{ST} in the simulated data set becomes significantly larger than F_{ST} as late as after 100 000 generations, supporting the finding that divergence between populations is not recent (see Results and the Supporting information). In addition, the mismatch analysis conducted on the whole sample set rejected the hypothesis of sudden population expansion, and no bottlenecks were observed in the whole microsatellite sample set or in the genetic groups. This is also congruent with the geological history of the area, which was a refuge during the recent ice ages and did not experience glaciations (Taberlet *et al.* 1998). The scenario of gene flow is also congruent with the IMa2 results (Fig. 5), which suggested migration of individu-

als among the six genetic groups. Further, the estimated split time between any tested pair of the six groups was larger than zero, pointing to mutual divergence among them, supporting the hypothesis of limited gene flow between diverged populations.

However, according to Gaggiotti (2011), the IM model assumes a constant level of migration from initial divergence to the present, meaning that temporal variations in migration might lead to biased results. Therefore, the migration rates estimated here could be an outcome of recent gene flow, migration during a restricted time-window or secondary contact (in this context understood as hypothetical transfer of fish performed by humans). The Geneclass2 results, assessing 16% of all individuals sampled in the present study as putative first-generation migrants, support the scenario of contemporary gene flow. The possibility of secondary contact cannot be excluded; though no records of fish transfer were found. As *D. adspersus* is of no commercial importance (Mrakovčić *et al*. 2006), their transplanting by humans is highly unlikely.

The hypothesis of recurrent gene flow between geographically isolated populations of *D. adspersus* thus appears to be the most likely explanation for the relatively low level of interpopulation genetic differentiation recorded here, and previously performed hydrological measurements and water tracing (Kanaet 1959; Bonacci 2006; Džeba 2010) support the hypothesis that the gene flow occurs via subterranean migration. With three cases discussed below, we present support for the hypothesis of recurrent underground migration.

The first case is provided by the groups Red Lake and 'central'. Red Lake is a sinkhole dating back to upper Miocene (Bahun 2000), with a water level well below the lip (Fig. 2), and hence completely excluding dispersion of fish by flooding. However, the existence of at least two underground canals connected to 'central' locations is known from the hydrological literature (Bonacci 2006), and our results are in line with those findings. Primarily, the prevailing haplotype in Red Lake was also most frequent in the 'central' group. In addition, BAPS analysis (Fig. 3) assigned most of the individuals from 'central' to the Red Lake group and the hierarchical STRUCTURE exhibits similarities between the two groups. Furthermore, there were two F_0 putative immigrants from 'central' present in Red Lake, while one individual migrated in the other direction. These numbers are small, but the method takes into account only the first-generation migrants, and the canal that connects Red Lake with the 'central' area is not active every year but only when precipitation is high (Bonacci 2006). Migration therefore probably does not occur in every generation. On the other hand, IM, which also detects past migration events, revealed higher migration rates, especially from Red Lake to 'central', supporting our hypothesis of underground gene exchange between these two groups.

The second example of probable underground migration is provided by *D. adspersus* inhabiting the superficially unlinked rivers MATR (Rastočka Matica) and MATV (Vrgoračka Matica; the southernmost locations). The fish from these two locations were identified as part of a single panmictic group, supported by hierarchical STRUCTURE, BAPS and *F*-statistics. MATV (Fig. 1) is completely isolated and has no surface connection to any other stream. Yet two individuals in MATR were designated as first-generation migrants from MATV, which reciprocally received three immigrants from MATR. In addition to the genetic evidence, dye tracing performed in 1954 revealed underground water connection between the rivers MATR and MATV (Kanaet 1959).

The third case supporting underground migration comes from the completely surface-separated 'central' and southernmost locations (Fig. 1). A genetic signature of 'central' is found in the southernmost locations and *vice versa* (BAPS; Fig. 3), while migration was also indicated by both Geneclass2 and IM. Considering only surface-water courses, 'central' formally belongs to the Vrljika river system, which ends in sinkhole Sajnoviči (Fig. 1). After passing under the Drinovci Mountain, part of the Vrljika River reappears as Tihaljina River, to which the southernmost locations are indirectly linked. Although the considerable difference in elevation between Vrljika and Tihaljina Rivers (90 vertical metres between sinkhole Sajnoviči and Peć—Mlini, where the Tihaljina River originates) could present an impassable upstream barrier for *D. adspersus*, our results imply the reverse. On the basis of dye tracing, there is evidence for underwater connections between Grude springs and the Tihaljina river system (Kanaet 1959) that seem to be connected via several estavelas (U-shaped siphons), which cause water to flow temporarily upstream (Jović 2003). This phenomenon is not rare in karst systems and may explain upstream migration of *D. adspersus*. Nevertheless, an alternative explanation is migration through some accessible underground connections. Based on hydrological measurements, Kanaet (1959) concluded that the Tihaljina River is supplied not only by the Vrljika River, but also by other, as yet undiscovered, underground sources, enabling fish migration.

There are at least 86 troglomorphic fish species in the world, many of them inhabiting karst environments in Australia, China, USA, Mexico, Thailand and the Middle East (Romero & Paulson 2001). Most of these species are comprised of strictly surface-dwelling, intermediate and completely stygobiontic populations that are often mutually isolated. In the example of *Astyanax mexicanus* and *Astyanax fasciatus* living in the karst areas of Central and North America (e.g. Mexico and

Florida), Strecker *et al.* (2004) suggested recurrent past invasions of caves, while surface populations sometimes became extinct. Thus, the complicated population structure identified is not a consequence of underground connections but rather a consequence of repetitive colonization from the surface. However, underground dispersal was speculated by Dillman *et al.* (2010), researching troglomorphic percopsiforms, who concluded that the present population structure probably reflects past connections in subsurface karst materials. Borowsky & Mertz (2001) found unique invasion with subsequent dispersion in the fish species *S. oedipus*, followed by isolation and differentiation of populations as a more probable explanation for the observed genetic structure than separate subterranean invasions of a common ancestor. Among epigenic fish species, underground dispersal has been proposed by Snoj *et al.* (2008) for softmouth trout (*S. obtusirostris*) in the Vrljika River. Even though in these cases underground migration is considered short-termed or a single event, the possibility of underground dispersal in well-developed karst systems observed by other researchers contributes to the same, but repeated, scenario that we propose for *D. adspersus*. In the case of this species, we infer continuous underground migrations as a feature of its biology, and this inference is supported by the species' morphology. While cavefish exhibit reduction in eyes, pigmentation and the swim bladder, the only possible adaptation to underground conditions observed in *D. adspersus* is a reduction in the number and complexity of scales (Mrakovčić *et al.* 2006). According to Freyhof *et al.* (2006), *Delminichthys* were trapped in this area by the rise of the Dinarids eight to ten million years ago. Therefore, we do not consider the lack of stygobiontic characters in *Delminichthys* a consequence of incomplete adaptation, but rather a consequence of the limited time that the species annually spends in the underground environment.

To the best of our knowledge, this study gives the first evidence of recurrent underground migration in epigenic fish and contributes to a better understanding of the biology and ecology of karstic ichthyofauna, as well as freshwater fish in general. In addition, it extends the knowledge of a vulnerable species that may establish a basis for its protection and contributes to the conservation strategy of the whole area. Because karst areas are widely distributed across the world, the findings are relevant to related fish species and other karst inhabitants and can contribute to conservation strategies for endemic karst organisms and karst ecosystems as a whole.

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References

Allouche S, Thevenet A, Gaudin P (1999) Habitat use by chub (*Leuciscus cephalus* L.1766) in a large river, the French Upper Rhone, as determined by radiotelemetry. *Archiv für Hydrobiologie*, **145**, 219–236.

Bahun S (2000) *O postanku Crvenog i Modrog jezera kot Imotskog. [On the origin of the Crveno and Modro Lakes near Imotski]*. Vodič ekskurzija - Excursion Guide-book, 2. Hrvatski geološki kongres, Zagreb, pp. 67–70.

Balloux F (2001) EASYPOP (Version 1.7): a computer program for population genetics simulations. *Journal of Heredity*, **92**, 301–302.

Becquet C, Przeworski M (2009) Learning about modes of speciation by computational approaches. *Evolution*, **63**, 2547–2562.

Bernatchez L, Wilson CC (1998) Comparative phylogeography of Nearctic and Palearctic fishes. *Molecular Ecology*, **7**, 431–452.

Bilopavlović V (1970) Gaovica u vodama Imotskog (Gaovica in waters of Imotski). *Priroda*, **57**, 221–222.

Bogutskaya NG, Zupančič P (2003) *Phoxinellus pseudalepidotus* (Telostei: Cyprinidae), a new species from the Neretva basin with an overview of the morphology of *Phoxinellus* species of Croatia and Bosnia-Herzegovina. *Ichthyological Exploration of Freshwaters*, **14**, 359–383.

Bonacci O (1987) *Karst Hydrology with Special Reference to Dinaric karst*. Springer-Verlag, New York, pp. 184.

Bonacci O (1993) Karst springs hydrographs as indicators of karst aquifers. *Hydrological Sciences Journal*, **38**, 51–62.

Bonacci O (2006) Crveno i Modro jezero kod Imotskog (Red and Blue Lakes near the town of Imotski). *Hrvatske vode – Časopis za vodno gospodarstvo*, **14**, 45–54.

Borowsky RB, Mertz L (2001) Genetic differentiation among populations of the cave fish *Schistura oedipus* (Cypriniformes: Balitoridae). *Environmental Biology of Fishes*, **62**, 225–231.

Corander J, Marttinen P (2006) Bayesian identification of admixture events using multi-locus molecular markers. *Molecular Ecology*, **15**, 2833–2843.

Corander J, Waldmann P, Sillanpää MJ (2003) Bayesian analysis of genetic differentiation between populations. *Genetics*, **163**, 367–374.

Corander J, Marttinen P, Mäntyniemi S (2006) Bayesian identification of stock mixtures from molecular marker data. *Fishery Bulletin*, **104**, 550–558.

Corander J, Marttinen P, Sirén J *et al.* (2008) Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics*, **9**, 539.

Detenbeck NE, DeVore PW, Niemi GJ *et al.* (1992) Recovery of temperate-stream fish communities from disturbance: a review of case studies and synthesis of theory. *Environmental Management*, **16**, 33–53.

Dillman CB, Bergstrom DE, Noltie DB *et al.* (2010) Regressive progression, progressive regression or neither? Phylogeny and evolution of the Percopsiformes (Teleostei, Paracanthopterygii). *Zoologica Scripta*, **40**, 45–60.

Džeba T (2010) *Određivanje otjecanja u slivu rijeke Ričine, Suvaje i Matice (Determination of runoff in the Ričina, Suvaja and Matica river basins)*. Master's Thesis, Faculty of Civil Engineering and Architecture, University of Split, Split.

Excoffier L (2007) Arlequin ver. 3.11 An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

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

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes*, **7**, 574–578.

Field MS (2002) *A Lexicon of Cave and Karst Terminology with Special Reference to Environmental Karst Hydrology*. USEPA, Washington, DC, pp. 194.

Franičević M, Tičina V (2003) Biometric characteristics of rare endemic fish, *Phoxinellus adspersu* (Heckel 1843), from Red Lake (Imotski, Croatia). *Periodicum Biologorum*, **4**, 453–460.

Freyhof J, Lieckfeldt D, Bogutskaya NG *et al.* (2006) Phylogenetic position of the Dalmatian genus *Phoxinellus* and description of the newly proposed genus *Delminichthys* (Teleostei: Cyprinidae). *Molecular Phylogenetics and Evolution*, **38**, 416–425.

Gaggiotti OE (2011) Making inferences about speciation using sophisticated statistical genetics methods: look before you leap. *Molecular Ecology*, **20**, 2229–2232.

Garašić M (2001) New speleohydrogeological research of Crveno jezero (Red Lake) near Imotskiin Dinaric karst area (Croatia, Europe). *Proceedings 13th International Congress of Speleobiology*, Brasilia, pp. 168–171.

Gavrilets S (2003) Perspective: models of speciation: what have we learned in 40 years? *Evolution*, **57**, 2197–2215.

Goudet J (2001) *Fstat, a Program to Estimate and Test Gene Diversities and Fixation Indices (version 2.9.3)*. Available from http://www.unil.ch/izea/softwares/fstat.html. University of Lausanne, Lausanne, Switzerland.

Gowans ARD, Armstrong JD, Priede IG *et al.* (2003) Movements of Atlantic salmon migrating upstream through a fish-pass complex in Scotland. *Ecology of Freshwater Fish*, **12**, 177–189.

Hardy OJ, Vekemans X (2002) SPAGeDi: a versatile compute program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, **2**, 618.

Hardy OJ, Charbonnel N, Freville H *et al.* (2003) Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. *Genetics*, **163**, 1467–1482.

Harpending HC (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology*, **4**, 591–600.

Hartl DL, Clark AG (1989) *Principles of Population Genetics*. Sinauer Associates, Inc., Sunderland, Massachusetts.

Hey J (2007) Introduction to the IM and IMa computer program. *Department of Genetics*, Rutgers University, pp. 3–4.

Hey J, Nielsen R (2004) Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. *Genetics*, **167**, 747–760.

Hey J, Nielsen R (2007) Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. *Proceedings of the National Academy of Sciences, USA*, **104**, 2785–2790.

Høgåsen HR (1998) Physiological changes associated with diadromous migration of salmonids. *Canadian Special Publication of Fisheries and Aquatic Sciences*, **127**, 128.

Hubisz MJ, Falush D, Stephens M *et al.* (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, **9**, 1322–1332.

Jović V (2003). *Vode zabiokovskog dijela neretvanskog sliva: istraženost i prijedlog daljnjih aktivnosti (Waters of Biokovo hinterland in Neretva watershead: research and future activity)*. Symposium *Voda u kršu Cetine, Neretve i Trebišnice*', Zbornik radova, Neum, 25–27, 18–30.

Kanaet T (1959) *Hidrografske prilike u slivu Tihaljina-Malde-Trebižat (Hydrographic characteristics in watershed of Tihaljina-Malde-Trebižat)*. Zbornik radova, 5. *Kongres geografa FNR Jugoslavije u NR Crnoj Gori* (Abstract book of geographic symposium in Yugoslavija, Montenegro), Cetinje, pp. 531.

Kranjc O (2004) Dinaric karst. In: *Encyclopedia of Caves and Karst Science* (ed. Gunn J), pp. 287–289. Fitzroy Dearborn, New York.

Kryštufek B, Buzan EV, Hutchinson WF *et al.* (2007) Phylogeography of the rare Balkan endemic Martino's vole, *Dinaromys bogdanovi*, reveals strong differentiation within the western Balkan Peninsula. *Molecular Ecology*, **16**, 1221–1232.

Li WH (1977) Distribution of nucleotide differences between two randomly chosen cistrons in a finite population. *Genetics*, **85**, 331–337.

Lucas MC, Baras E (2001) *Migration of Freshwater Fishes*. Blackwell Science Ltd, Oxford, pp. 420.

Matschiner M, Salzburger W (2009) Tandem: integrating automated allele binning into genetics and genomics workflows. *Bioinformatics*, **25**, 1982–1983.

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure from human nucleated cells. *Nucleic Acids Research*, **16**, 1215.

Mrakovčić M, Brigić A, Buj I, *et al.* (2006) *Crvena knjiga sladkovodnih riba hrvatske (Red book of freshwater fish of Croatia)*. Ministrstvo kulture, Državni zavod za zaštitu prirode, Republika Hrvatska.

Nadachowska K, Babik W (2009) Divergence in the face of gene flow: the case of two newts (Amphibia: Salamandridae). *Molecular Biology and Evolution*, **26**, 829–841.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Niemiller ML, Fitzpatrick BM, Miller BT (2008) Recent divergence with gene flow in Tenessee cave salamanders (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. *Molecular Ecology*, **17**, 2258–2275.

Paetkau D, Slade R, Burdens M, *et al.* (2004) Genetic assignment methods for the direct, real-time estimation of migration rate; a simulation-based exploration of accuracy and power. *Molecular Ecology*, **13**, 55–65.

Palandačić A, Zupančič P, Snoj A (2010) Revised classification of former genus *Phoxinellus* using nuclear DNA sequences. *Biochemical Systematics and Ecology*, **38**, 1069–1073.

Perea S, Bohme M, Zupančič P *et al.* (2010) Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evolutionary Biology*, **10**, 265.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **86**, 502–503.

Piry S, Alapetite A, Cornuet JM *et al.* (2004) GENECLASS2: a software for genetic assignment and first-generation migrant detection. *Journal of heredity*, **95**, 536–539.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Romero A, Paulson KM (2001) It's a wonderful hypogean life: a guide to the troglomorphic fishes of the world. *Environmental Biology of Fishes*, **62**, 13–41.

Schneider S, Excoffier L (1999) Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, **152**, 1079–1089.

Sket B, Paragamian K, Trontelj P (2004) A census of the obligate subterranean fauna of the Balkan Peninsula. In: *Balkan Biodiversity, Pattern and Process in the European Hotspot* (eds Griffiths HI, Kryštufek B and Reed JM), pp. 309–322. Kluwer, Dordrecht.

Slatkin M, Excoffier L (1996) Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. *Heredity*, **76**, 377–383.

Snoj A, Bogut I, Sušnik S (2008) Evidence of a genetically distinct population of Vrljika softmouth trout *Salmo obtusirostris* Heckel evolved by vicariance. *Journal of Fish Biology*, **72**, 1945–1959.

Strasburg JL, Rieseberg LH (2011) Interpreting the estimated timing of migration events between hybridizing species. *Molecular Ecology*, **20**, 2353–2366.

Strecker U, Faúndez VH, Wilkens H (2004) Phylogeography of surface and cave *Astyanax* (Teleostei) from Central and North America based on cytochrome *b* sequence data. *Molecular Phylogenetics and Evolution*, **33**, 469–481.

Swofford DL (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, ed. 4.04a. Sinauer Associates, Sunderland, Massachusetts.

Taberlet P, Fumagalli L, Wust-Saucy AG *et al.* (1998) Comparative phylogeography and postglacial colonization routs in Europe. *Molecular Ecology*, **7**, 453–464.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Likelihood, Distance, and Parsimony methods. *Molecular Biology and Evolution*, **12**, 2731–2739.

Vähä JP, Erkinaro J, Niemelä E *et al.* (2007) Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. *Molecular Ecology*, **16**, 2638–2654.

Van Oosterhout C, Hutchinson WF, Wills DPM *et al.* (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Verovnik R, Sket B, Trontelj P (2004) Phylogeography of subterranean and surface populations of water lice *Asellus aquaticus* (Crustacea: Isopoda). *Molecular Ecology*, **13**, 1519–1532.

Weir BS, Cockerheim CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Zardoya R, Doadrio I (1999) Molecular Evidence on the Evolutionary and Biogeographical Patterns of European Cyprinids. *Journal of Molecular Evolution*, **49**, 227–237.

Zupančič P (2008) *Rijetke I ugrožene slatkovodne ribe jadranskog slijeva Hrvatske, Slovenije i Bosne i Hercegovine (Rare and endangered freshwater fishes of Croatia, Slovenia and Bosnia and Hercegovina)*. AZV, Dolsko, pp. 79.

Zupančič P, Bogutskaya NG (2000) Description of a new species, *Phoxinellus dalmaticus* (Cyprinidae: Leuciscinae), from the Čikola River in the Krka River system, Adriatic basin (Croatia). *Natura Croatica*, **9**, 67–81.

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Data accessibility

DNA sequences: GenBank accessions JN101952–JN102110 and JQ315936–JQ316113. Raw microsatellite data, tandem output file and IMa2 input files are available in the supplementary material on the *Molecular Ecology* web site.

Supporting information

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

Data S1 Materials and Methods.

Data S2 Results.

Data S3 Geneclass2 output file.

Data S4 Raw microsatellite data.

Data S5 Tandem output file.

Data S6 IMa2 input files.

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