JOURNAL OF Evolutionary Biology
eseb European Society for Evolutionary Biology

doi: 10.1111/jeb.12610

Parallels between two geographically and ecologically disparate cave invasions by the same species, *Asellus aquaticus* (Isopoda, Crustacea)

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Keywords:

adaptation;
divergence;
microsatellites;
morphometric traits;
parallel evolution;
population structure;
subterranean;
troglomorphy.

Abstract

Caves are long-known examples of evolutionary replications where similar morphologies (troglomorphies) evolve independently as the result of strong natural selection of the extreme environment. Recently, this paradigm has been challenged based on observations that troglomorphies are inconsistent across taxa and different subterranean habitats. We investigated the degree of replicated phenotypic change in two independent cave invasions by the freshwater isopod *Asellus aquaticus*; the first in a sulphidic aquifer in Romania, the second in a sinking river in the Dinaric Karst in Slovenia. Both ancestral surface populations still live alongside the subterranean ones. Phylogenetic analyses show independence of the two colonization events, and microsatellite analysis shows no evidence of ongoing genetic exchange between surface and subterranean ecomorphs. The overall morphology has changed dramatically at both sites (50 of 62 morphometric traits). The amount of phenotypic change did not reflect differences in genetic diversity between the two ancestral populations. Multivariate analyses revealed divergent evolution in caves, not parallel or convergent as predicted by the current paradigm. Still, 18 traits changed in a parallel fashion, including eye and pigment loss and antennal elongation. These changes might be a consequence of darkness as the only common ecological feature, because Romanian caves are chemoautotrophic and rich in food, whereas Slovenian caves are not. Overall, these results show that morphologically alike surface populations can diverge after invading different subterranean habitats, and that only about one-third of all changing traits behave as troglomorphies in the traditional sense.

Introduction

Cases of dramatic replicated evolutionary change associated with the colonization of new habitats contain instructive lessons on the deterministic nature of natural selection (Darwin, 1859; Schluter, 2000; Mahler *et al.*, 2013). Caves are examples of such new habitats that are particularly renowned for yielding numerous consistent phenotypic similarities, termed troglomorphies (Christiansen, 1962; Culver *et al.*, 1995). According to the predominant paradigm of subterranean biology, troglomorphies are the result of strong natural selection in the extreme subterranean environment. This concept has remained popular to the present time (Soares & Niemiller, 2013; Gross *et al.*, 2014; Yu *et al.*, 2014). However, it has been challenged by Pipan & Culver (2012) who argued that (i) the same cave-related morphologies can be found not only in caves but also in shallow subterranean habitats that are richer in nutrient than caves and (ii) permanent subterranean dwellers do not always evolve such traits. They propose that these inconsistencies in phenotypic change can be

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best explained by variation among different types of subterranean environments. Varying ecological and historical factors include influence of diurnal and annual cycles, food availability, time since colonization and the mode of colonization. Only the absence of light is universal to all settings. This leads to the rather radical conclusion that darkness alone is the major unifying selective force responsible for phenotypic similarity across subterranean species in various habitats (Culver *et al.*, 2010).

This 'darkness-as-major-selective-force' hypothesis implies that troglomorphies in the sense of general adaptations to the subterranean environment are too loosely defined. As such they cannot serve as those indisputable proofs of the deterministic action of natural selection envisaged by Darwin (1859). Even though some reduced traits such as eye loss and depigmentation regularly accompany life in total darkness, it is the elaborated traits, such as elongation of appendages or augmentation of extra-optical sense organs that are most intriguing. For example, if one cave species evolves long antennae and another one not, an *ad hoc* explanation is needed to uphold the validity of the hypothesis that long antennae are an adaptation to the subterranean environment.

One possible solution is narrowing down the concept of troglomorphy to those convergent changes that are common across species in different types of subterranean environments. However, convergence, defined as similar change affecting different species of separate ancestry (Conte *et al.*, 2012), is influenced by phylogeny and taxon-specific factors (Losos *et al.*, 1998; Langerhans *et al.*, 2006). Less prone to these influences is parallel evolution, that is similar changes occurring in populations evolving from the same ancestor. Nevertheless, nonconvergent or nonparallel evolution has been found by most studies addressing parallel evolution in independent contrasts within species (Eroukhmanoff *et al.*, 2009; Kaeuffer *et al.*, 2012).

The study system we present here takes these considerations into account. We were interested in the degree of replicated phenotypic change between supposedly independent cave invasions by the freshwater isopod *Asellus aquaticus* Linné, 1758 that took place in a sulphidic aquifer in Romania and in a sinking river in the Dinaric Karst in Slovenia (Sket, 1994; Turk-Prevorčnik *et al.*, 1996; Turk-Prevorčnik & Blejec, 1998; Verovnik *et al.*, 2004) (Fig. 1). The two subterranean habitats lie 1200 km apart and differ in nearly all ecological factors except for darkness. Ecological characteristics of both habitats are described in Appendix S1. At both sites, the ancestral surface population still lives alongside and in occasional sympatry with the subterranean one that has evolved into a new, troglomorphic ecomorph. Factors such as available time or the amount of ancestral genetic variation are potentially important (Kaeuffer *et al.*, 2012) and were controlled for by analysis of mitochondrial and microsatellite DNA.

Our rationale behind this comparative analysis was that traits changing in the same way in the Romanian and the Slovenian pair of ecomorphs despite ecological and historical differences are potential adaptations to the complete lack of light as the only known common feature of both subterranean environments. Thus, they are troglomorphies in the narrow sense (e.g. sensu Pipan & Culver, 2012). Specifically, our aims were (i) to test whether the subterranean ecomorphs in Romania and Slovenia evolved independently, (ii) to compare genetic diversity and structuring between both surface–subterranean pairs of populations and (iii) to provide a quantitative framework for the assessment of morphological evolution in the subterranean environment.

Materials and methods

Samples

Surface and subterranean ecomorphs from Romania had not yet been analysed genetically, so our first goal was to assess their phylogenetic position and to test whether the two cave populations indeed represent independent colonizations of subterranean habitats. For that purpose, we collected animals from surface (52 individuals) and subterranean habitats (82 individuals) from Dobrogea, Romania (Fig. 1, Table 1). We added data from a phylogeographical study of *A. aquaticus* across Europe (Verovnik *et al.*, 2005), as well as a few new localities from south-eastern Europe (Appendix S2). The Slovenian data set included DNA sequences from Verovnik *et al.* (2004) supplemented by new specimens from several localities in the Postojna-Planina Cave System (120 individuals) and adjacent surface localities on the Planina Polje (70 individuals) (Fig. 1, Table 1, Appendix S2).

For the analysis of morphological traits in both pairs of ecomorphs, samples and data from previous research (Prevorčnik *et al.*, 2004) were used. About 20 adult males were collected in the 1990s at each of four sites as explained in Table 1 (details in Appendix S1).

DNA extraction and amplification

Genomic DNA was isolated from whole animals or just from the sixth pair of pereopods, so that the morphology of specimens was preserved. We used the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) following the mammalian tissue preparation protocol.

For each of the specimens, a 653-bp fragment of the first subunit of cytochrome oxidase mitochondrial gene (COI) was amplified according to the protocol described in Verovnik *et al.* (2004). The nuclear 28S rRNA gene (750-bp long) was amplified using primers and protocol

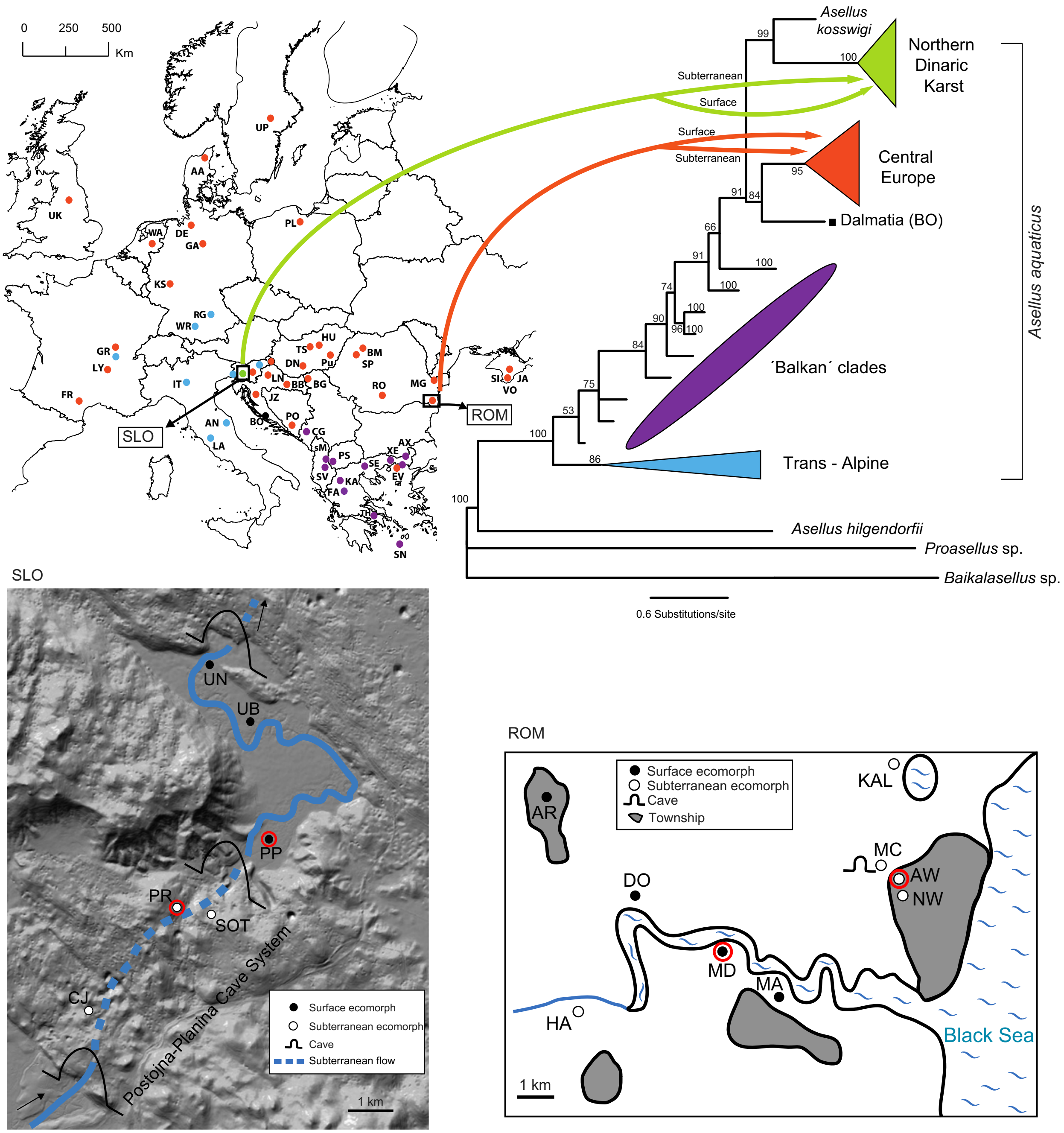

Fig. 1 Global phylogeographic situation of *Asellus aquaticus* based on Bayesian phylogeny of mitochondrial (COI) haplotypes, and map of study sites in Slovenia (SLO) and Romania (ROM). Sampling sites are denoted by a two-letter code as per Table 1 with further details about geographic coordinates and DNA sequences given in Appendix S2. Specimens used in morphometric analyses were taken from the sites marked with red circles. The tree is rooted on the asellid outgroups *Proasellus* and *Baikalasellus*. Numbers on branches are Bayesian posterior probabilities.

from Verovnik *et al.* (2005). As the 28S rRNA gene is not as polymorphic as the COI gene, a smaller number of samples were used (see Appendix S2 for details).

We also analysed DNA length polymorphism at eight polymorphic microsatellite loci: A2, A9, A110, A111, A234, A236, A262 and C6 (details regarding primers and PCR conditions are provided in Appendix S2). Four of these loci (A234, A262, A236, and C6) were newly developed in this study, the rest have been already published (Molecular Ecology Resources Primer Devel-

Table 1 Sampling sites and data sources of *Asellus aquaticus* specimens from Romania and Slovenia.

Sampling site*	Code	Ecomorph	Sample size		
			mtDNA	Microsatellites	Morphology
Romania					
Neţoi Street, hand-dug well	NW	Subterranean	31	30	
D. Ana Street, hand-dug well	AW	Subterranean	10	12	22
Movile Cave	MC	Subterranean	3	3	
Karaoban Lake	KAL	Subterranean	30	31	
Hagieni, sulphur spring	HA	Subterranean	3	6	
Limanu Lake shore	MD	Surface	14	18	20
	MA	Surface	5	9	
	DO	Surface	12	17	
Arsa village, freshwater well	AR	Surface	8	8	
Slovenia					
Postojna-Planina Cave System	PR	Subterranean	20	78	24
	SOT	Subterranean	11	37	
	CJ	Subterranean	5	5	
PlaninaPolje	PP	Surface	10	32	24
	UB	Surface	9	26	
	UN	Surface	10	12	

*The geographic position of each site is shown on Fig. 1.

opment Consortium *et al.*, 2011). PCR products containing the microsatellites were separated on an Applied Biosystems 3130xl Genetic Analyzer. Results were analysed in GENEMAPPER 4.0 [Applied Biosystems, part of LifeTechnologies (a Thermo Scientific brand), Foster City, CA, USA)]. DNA sequences are deposited in GenBank, accession numbers are listed in Appendix S2.

Analyses based on DNA sequences

DNA sequences were edited in Geneious 5.04 (Biomatters Ltd., Auckland, New Zealand). All COI sequences were the same length and hence aligned unambiguously. The correctness of the alignment was verified at the amino acid level using the invertebrate mitochondrial genetic code. Only unique haplotypes were used for phylogenetic tree construction. All new sequences were added to the large European *Asellus* COI data set (Verovnik *et al.*, 2005). Only the Central European group of haplotypes identified in that study was used for further analyses, once it was established that all sequences of interest are part of this clade. Nuclear 28S rRNA gene sequences were gap-free and aligned by hand to the alignment from Verovnik *et al.* (2005).

To visualize and compare the patterns of diversification of both subterranean populations, COI median-joining networks (Bandelt *et al.*, 1999) were constructed based on 214 Romanian and 65 Slovenian COI haplotypes. To calculate the most parsimonious network (Steiner tree), the software Network 4.610 (www.fluxus-engineering.com) was used. Equal weights were assigned to all positions, and the homoplasy level parameter (ε) was set to zero.

In the phylogenetic analyses, 28S rRNA gene sequences were used in addition to the COI sequences. Both data sets were treated separately, as they have different levels of genetic variation. The more conserved 28S rRNA gene was analysed only for a subset of individuals to verify the deep structure of the mitochondrial topology. Bayesian phylogenetic tree searches were run under MrBayes 3.2 (Ronquist *et al.*, 2012). The optimal substitution model was selected according to the Akaike information criterion (AIC) in JMODELTEST 0.1.1 (Posada, 2008). An HKY85 (Hasegawa–Kishino–Yano) model of nucleotide substitution with gamma distributed rate heterogeneity and a significant proportion of invariable sites was used for the COI sequence data. For the 28S rRNA data, a general time-reversible (GTR) model of nucleotide substitution with gamma distributed rate heterogeneity was selected. A Markov chain Monte Carlo (MCMC) search was run with two simultaneous runs of four chains for 2×10^6 generations, taking samples every 100 generations. The first 25% of trees were discarded as burn-in.

Nucleotide diversity (π) was calculated in DNAsp 5.10 (Librado & Rozas, 2009). Analysis of molecular variance (AMOVA) was conducted to compare the proportion of genetic variation within and between ecomorphs for both the Romanian and the Slovenian pairs. Individuals were grouped according to their ecomorph affiliation. The program Arlequin 3.5.1.2 (Excoffier & Lischer, 2010) was used to perform the analyses.

We estimated the divergence times of subterranean and surface ecomorphs using the coalescent approach in BEAST 1.6.2 (Drummond *et al.*, 2007). A HKY85 substitution model with gamma distributed rate of substitution and significant number of invariable sites was used. We applied a relaxed clock model (Drummond *et al.*, 2006) and used a known mutation rate of 0.0125 substitutions per site per million years that had been estimated for the mitochondrial COI gene in the subterranean isopod genus *Stenasellus* (Ketmaier *et al.*, 2003). This rate has already been shown to yield reasonable estimates in *A. aquaticus* (Verovnik *et al.*, 2005). Constant population size was assumed, and MCMC chains of 10^6 steps were run under default priors. Results were analysed in Tracer 1.5 (Rambaut & Drummond, 2007), making sure the effective sample size was high enough for age estimates of the chosen taxon sets.

Microsatellite analyses

The software Convert 1.31 (Glaubitz, 2004) was used to convert the data into different types of files for subsequent use. Number of alleles, observed and expected heterozygosity (H_o, H_e), tests for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated in Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). Between- and within-ecomorph partitioning of genetic variance was calculated applying AMOVA in the same manner as was carried out with COI haplotypes.

The genetic population structure was explored using the Bayesian program Structure 2.3.3 (Pritchard *et al.*, 2000) in which individuals are assigned to populations using estimated allele frequencies. A model allowing admixture without any *a priori* information was run 15 times for number of clusters *K* from 1 to 10 on the Romanian data set and for *K* from 1 to 7 on the Slovenian data set. The same was repeated with the LOCPRIOR model, which should better detect weak structure, if present. The optimal number of clusters (*K*) for our data was determined using Structure Harvester Web v0.6.92 (Earl & vonHoldt, 2012) using the Evanno method (Evanno *et al.*, 2005). The Bayesian method implemented in Structure is sensitive to deviations from Hardy–Weinberg equilibrium, linkage disequilibrium (Pritchard *et al.*, 2000; Rodríguez-Ramilo *et al.*, 2009) and inbreeding. Any of these factors can result in overestimating the number of clusters (Hubisz *et al.*, 2009; Rodríguez-Ramilo *et al.*, 2009). Therefore, we assessed population structure also using the program Cluster 1.0 (Rodríguez-Ramilo *et al.*, 2009), where the implemented criterion is the maximization of the averaged genetic distance between a predefined number of clusters and which does not make any assumptions based on Hardy–Weinberg equilibrium and linkage disequilibrium. The number of tested clusters ranged from two to seven. We calculated the highest rate of change in the averaged genetic distance between successive *K* values (ΔK) to find the optimal solution (Rodríguez-Ramilo *et al.*, 2009). Finally, we used discriminant analysis of principal components (DAPC, Jombart *et al.*, 2010), which is unaffected by deviations from Hardy–Weinberg and linkage equilibrium, to supplement the results of the previous genetic structure analyses. The optimal number of genetic clusters was identified using the 'find.clusters' function implemented in adegenet (Jombart, 2008), which first transforms the data using principal component analysis (PCA) and then looks for the optimal number of clusters based on Bayesian information criterion. The analysis was run in R 3.1.2 (R Development Core Team, 2014).

Analysis of morphometric traits

To explore the morphological variation within and between ecomorphs, we analysed all 60 morphometric traits know so far to independently vary between different *A. aquaticus* populations (Prevorčnik *et al.*, 2004). First, we conducted a multivariate analysis, exploring the overall pattern of morphological change. The shift in morphospace of the subterranean ecomorph relative to their surface ancestors was visualized using PCA performed on a correlation matrix using all traits except the presence/absence characters (eyes, pigmentation).

The second approach was a univariate trait-by-trait comparison of the amount and direction of change between surface and subterranean ecomorphs. This comparison can reveal several patterns: no significant change, significant change of mean and significant change of variance. The underlying assumptions are that (i) the change occurred in the subterranean population when or after it invaded its new habitat and (ii) the trait remained unchanged in the ancestral surface population or has changed much less than in the subterranean population. In the pairwise comparison of the Slovenian and the Romanian ecomorphs, 26 numerical counted traits and 32 ratios (calculated from metric traits) from Prevorčnik *et al.* (2004) were included along with two additional traits: body length and relative body width (BWL) (Appendix S3). The latter trait was used as a measure of overall body shape instead of previously used relative width of pereomere V (P5 in Prevorčnik *et al.*, 2004). Two-sample *t*-tests with control for false discovery rate (FDR) set to $q = 0.001$ for 60 tests were used for comparison of means. Homogeneity of variance was tested using Levene's statistic with FDR control set to $q = 0.01$ for 60 tests. Body pigmentation (BP) and the presence of eyes (E) displayed no within-ecomorph variability and were treated as present/absent only. All analyses of morphometric data were conducted in IBM SPSS 20.0 for Windows (2011) (IBM Corp., Armonk, NY, USA).

A heatmap was constructed to visualize the global pattern of trait change in the two subterranean ecomorphs. As basis for the heatmap served a matrix with relative change (as compared to the respective surface population means) in per cent with individuals as rows and traits as columns. Colour intensity was calculated in correspondence to the amount of change using the conditional formatting function in Microsoft Excel 2013.

Morphometric data are available on Dryad.

Results

Independent origin of subterranean ecomorphs

Phylogenetic and network analyses

In the mitochondrial DNA phylogeny (Fig. 1), all samples from Romania, including the Romanian subterranean ecomorph, were nested within a larger Central Europe clade. By contrast, Slovenian surface and subterranean samples were part of a Northern Dinaric Karst clade that is phylogenetically distinct from the Central Europe clade (see Verovnik *et al.*, 2005 for Europe-wide phylogeography of *A. aquaticus*). The phylogenetic distance and the large geographic distance speak strongly in favour of independent cave colonization events in Slovenia and Romania. In both areas, the subterranean populations were genetic subgroups of the surface populations from the local area and are younger than the splits among the main European phylogeographic groups. This result was supported also by the nuclear gene phylogeny (Appendix S4).

It is noteworthy that mitochondrial DNA clades of *A. aquaticus* form a paraphyletic assemblage through the exclusion of *Asellus kosswigi*. However, individuals from different clades interbreed freely in areas of secondary contact, whereas *A. kosswigi* and *A. aquaticus* do not interchange genes in sympatry (Verovnik *et al.*, 2005).

A two-fold origin of the subterranean ecomorph was apparent in the Romanian mitochondrial haplotype network (Appendix S4). Two separate groups of subterranean haplotypes were embedded between numerous other Central European haplotypes. Also, the ancestral haplotypes might already be extinct as their positions were replaced by median vectors (hypothetical ancestral haplotypes) in the network of the Central Europe clade. Conversely, in the subterranean part of the Slovenian network, no missing haplotypes were identified. A star-like pattern of haplotypes separated by one mutation step suggested a recent colonization and expansion into the subterranean habitat. Ancestral haplotypes were still present and very common, shared by both Slovenian ecomorphs.

Age of colonization

Using relaxed clock dating, the time to the most recent common ancestor was estimated to be between 1.3 and 0.2 Mya in the Slovenian, and between 3.8 and 0.2 Mya in Romanian subterranean ecomorphs. These results suggest that *A. aquaticus* colonized the subterranean habitat some time during the climatically turbulent Pleistocene epoch, in Romania possibly earlier than in Slovenia. Because of the large intervals, we were not able to use these data to test for correlation with morphological evolution.

Comparative population genetics of subterranean ecomorphs

Genetic population structure

We analysed the genetic structure in the Romanian and Slovenian pair of ecomorphs using eight microsatellite loci. In both pairs, DAPC (Jombart *et al.*, 2010) and Structure (Pritchard *et al.*, 2000) assigned individuals to two clusters that were in complete concordance with ecomorph status (Fig. 2). Ancestral surface populations from Slovenia and Romania were genetically completely separated from each other. In contrast to mtDNA data, microsatellites indicated that the Slovenian subterranean population was more strongly differentiated from its surface ancestor than the Romanian one. All individuals were assigned to their respective clusters with $q > 0.95$ (q being the estimated membership coefficient), except for one surface individual from Romania with a slightly lower $q = 0.88$. This shows there is no admixture (Beaumont *et al.*, 2001; Vähä & Primmer, 2006) between the subterranean and the surface ecomorphs in both systems. We obtained the same result by the distance-based approach implemented in Cluster (Rodríguez-Ramilo *et al.*, 2009) that does not depend on assumptions about Hardy–Weinberg equilibrium and linkage disequilibrium. The subterranean forms thus represent independent genetic entities.

When we analysed each local ecomorph separately, we found no substructure in Slovenian samples. However, in both Romanian ecomorphs, individuals were assigned to three clusters ($K = 3$), most of them in concordance with their sampling localities. This substructuring also explains deviations from Hardy–Weinberg equilibrium and linkage disequilibrium found in both Romanian ecomorphs (see also Table 2). Within these subgroups, most loci were in Hardy–Weinberg equilibrium or monomorphic. All microsatellite data are available on Dryad.

Partitioning of molecular variance

A fundamental point of comparison was how genetic variability is distributed between and within ecomorphs. AMOVA gave contrasting results for the Romanian and Slovenian pair of ecomorphs. In the Romanian pair of ecomorphs, variance components were well-balanced regardless of the marker system used (49% between and 51% within for mtDNA; 40% between and 60% within for microsatellites). Conversely, Slovenian ecomorphs showed much higher within-ecomorph variation of mitochondrial DNA (78%), and a much lower within-ecomorph variation (13%) of microsatellite DNA. Given that microsatellites are a faster evolving marker system than mitochondrial

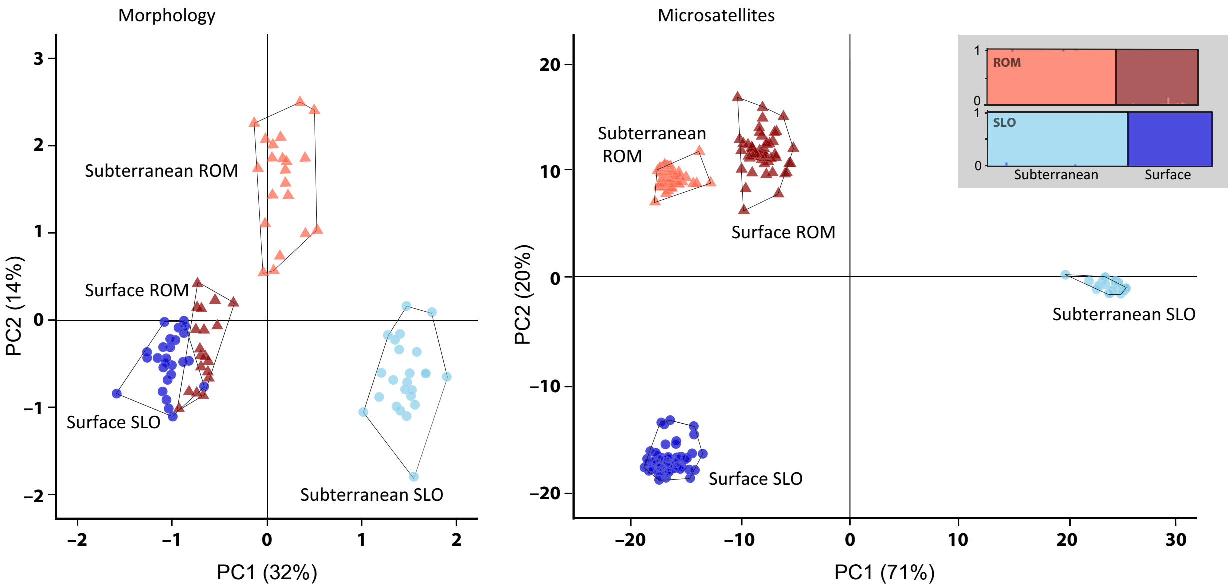

Fig. 2 Principal component analyses of morphometric traits (left plot) and microsatellite data (right plot) in surface/subterranean ecomorph pairs of *Asellus aquaticus*. Both morphologically and genetically, Romanian (ROM) and Slovenian (SLO) subterranean populations are well separated from their respective ancestral surface populations. Genetic isolation between subterranean and surface forms is further indicated by unambiguous clustering of all subterranean and surface individuals in the structure plot (shaded box).

Table 2 Genetic diversity of surface and subterranean *Asellus aquaticus* ecomorphs.

	Mitochondrial DNA		Microsatellites		
	N	π	*N*	H_e	H_o
Romania					
Subterranean	77	0.018	82	0.34	0.19
Surface	39	0.017	52	0.69	0.34
Slovenia					
Subterranean	36	0.0025	120	0.053	0.052
Surface	29	0.0035	70	0.25	0.15

N, number of analysed individuals; π, nucleotide diversity; H_e, expected heterozygosity; H_o, observed heterozygosity.

DNA, this finding could be an indication of younger age of the Slovenian subterranean ecomorph.

Genetic diversity

A comparison of standard genetic diversity indices for mitochondrial DNA and microsatellites revealed a common trend for both ecomorph pairs. Subterranean ecomorphs lost one-third to one half of their heterozygosity compared to their ancestral surface ecomorph (Table 2). Diversity of mitochondrial DNA remained roughly unchanged. Another observation was that most diversity indices were considerably lower in Slovenia than in Romania. This is in accordance with the polyphyly of subterranean mitochondrial DNA haplotypes in Romania.

Morphological change

Multivariate analysis

On the PCA plot, the Romanian and the Slovenian surface populations were grouped closely together, whereas both subterranean populations are clearly distinct from their surface ancestors and from each other (Fig. 2). The first principal component (32% of total trait variance) separated the subterranean ecomorphs from their surface ancestors. It received the highest loading by variation in relative length and flagellar article number of antenna II (A2, A2N), relative lengths of pereopod VII (PE7) and uropod (U), as well as certain setae number features: uropod endopodite setae number (USN) and setae number on dorsal margins of pereopods IV and VII protopodite (PE45SU, PE75SU). Both subterranean ecomorphs were well separated also along the second principal component (14% of total trait variance) with the highest loading by setae number along pleotelson margin (PTS).

Trait-by-trait analysis

Of 62 traits, 42 differed significantly in the Slovenian and 28 in the Romanian pair of ecomorphs (Appendix S3). According to the observed differences, traits were divided into five classes (Fig. 3):

1 Traits showing significant unidirectional change in both ecomorph pairs: 18 traits. Subterranean ecomorphs have, besides lacking body pigmentation and eyes (BP, E): a reduced number of antennae I articles

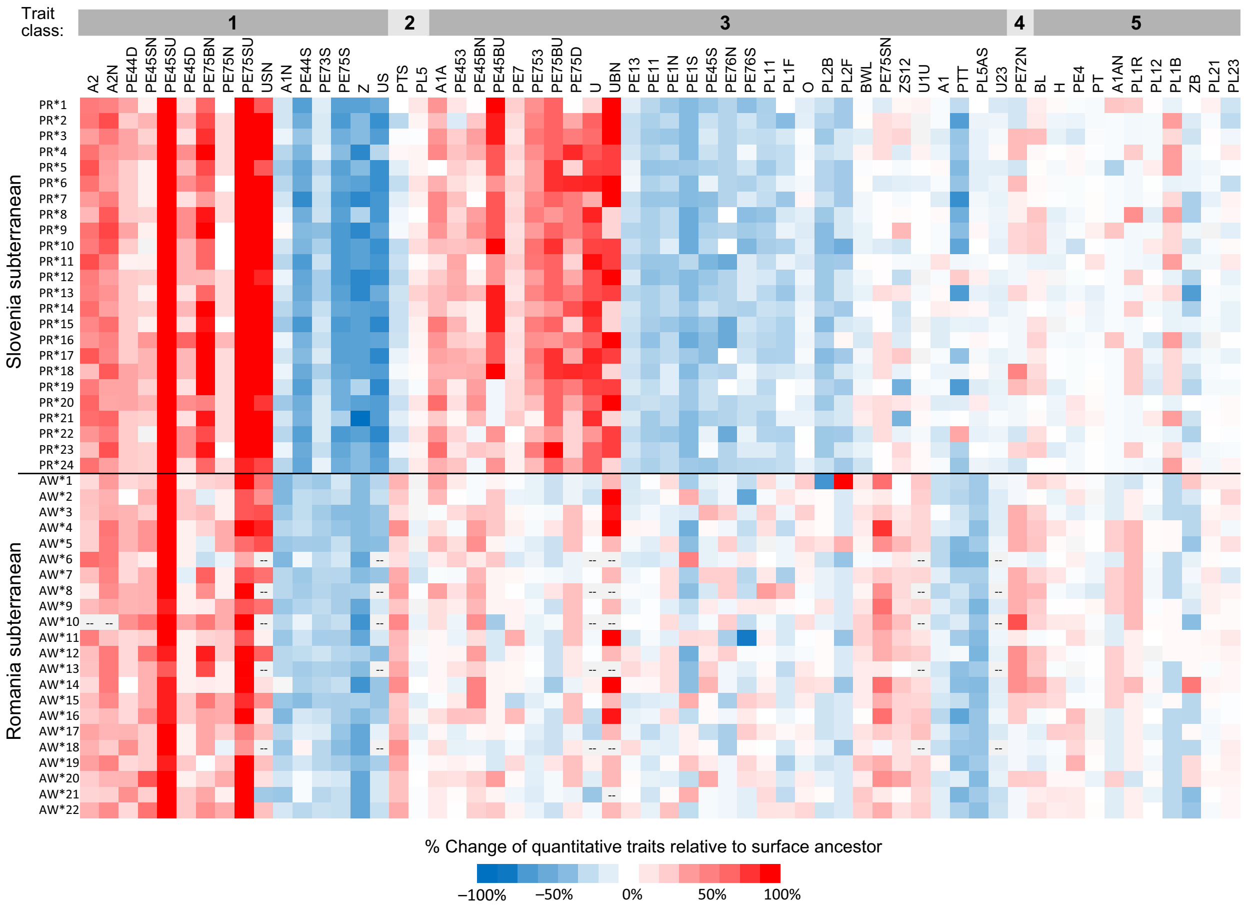

Fig. 3 Heatmap showing the pattern of quantitative trait change in Slovenian and Romanian subterranean *Asellus aquaticus*. Individual change in trait values in the subterranean ecomorphs relative to surface ancestor population average in per cent is depicted as colour ranging from dark blue (−100%) over white (no change) to dark red (+100%). Traits are grouped by classes: (1) traits changing significantly in the same direction in both ecomorphs; (2) traits changing significantly in opposing direction in both ecomorphs; (3) traits changing significantly in only one ecomorph; (4) traits with significant change in variance but unchanged mean in one or both ecomorphs (PE1N had increased variance in Romania, but changed significantly in Slovenia); (5) traits with no significant change in neither mean nor variance. Trait symbols are listed and explained in Appendix S3. Eyes and body pigment were treated as qualitative traits and therefore not included in the table. Missing data are indicated by dashes. Individual specimens are marked by their respective locality code: PR for Postojna-Planina Cave System, AW for D. Ana Street hand-dug well (see also Fig. 1 and Table 1).

(A1N), shortened certain simple setae on pereopods IV, VII and uropod (PE44S, PE73S, PE75S, US), as well as a more shallow concavity on the pleopod I exopodite (Z). They have elongated antennae II (A2) with more flagellar articles (A2N) and an increased number of certain simple setae on pereopods IV, VII and uropod (PE44D, PE45D, PE45SN, PE45SU, PE75BN, PE75N, PE75SU, USN). The listed traits are candidates for narrow-sense troglomorphies and thus for further examination of their adaptive function with respect to general aspects of the subterranean environment.

2 Traits showing significant opposing change in both ecomorph pairs: two traits. Number of setae along pleotelson margin (PTS) is decreased in the Slovenian subterranean ecomorph and increased in the Romanian one, whereas the situation is reversed in relative width of pleopod V (PL5). These traits could point to specific aspects of the subterranean environment in Romania and Slovenia.

3 Traits that changed in one but not in the other ecomorph pair: 30 traits (22 in the Slovenian and 8 in the Romanian pair). The interpretation of these traits is inconclusive; different combinations of the above scenarios and neutrality with respect to the subterranean environment may apply.

4 Traits showing significant change in variance, but with unchanged mean values: two traits in the Romanian ecomorph pair, referring to the number of certain setae on pereopods (PE1N, PE72N). As trait variance is significantly higher in the subterranean ecomorph than in the surface form, these are likely candidates for traits experiencing release of selection in the subterranean environment.

5 Traits in which neither mean values nor variances have changed: 11 traits.

Summarized in the heatmap on Fig. 3, a global pattern of trait evolution emerges. It shows that about one quarter of all traits changed in a convergent or parallel way, and about one half of all traits changed only in one of the two subterranean ecomorphs. Traits changing in different directions in both ecomorphs, as well as traits with increased variance but unchanged mean, are scarce. About one-fifth of all traits did not experience any significant change. Strong colours predominate in the Slovenian subterranean ecomorph, indicating greater overall morphological change than in the Romanian one. Red and blue tones are proportionate, suggesting that there is no general trend towards increase or decrease in size or number of quantitative traits in caves.

Discussion

We present a new study system with two replicated pairs of known ancestor and descendant populations that colonized a novel habitat. The freshwater isopod *A. aquaticus* shifted to its subterranean habitat twice independently in different parts of Europe and under different historical circumstances and ecological set-ups. A multifaceted approach allowed us to examine how historical and current ecological factors, genetic variation and ancestral conditions influence the anticipated convergent or parallel evolutionary changes.

Different genetic diversity patterns reflect ecological and geographical setting but do not affect morphological evolution

Comparisons of genetic diversity revealed both concordant and contrasting patterns between the Romanian and the Slovenian system. The subterranean ecomorphs in Romania and Slovenia are substantially less diverse genetically than their respective ancestral surface populations. On average, variation between ecomorphs accounts for about one half of the total genetic variation. High differentiation from the ancestor and a decline in genetic diversity in caves is a pattern frequently predicted as a consequence of founder effect or periodic bottlenecks caused by fluctuating water levels and food scarcity, or smaller population size *per se* (Strecker *et al.*, 2003; Carlini *et al.*, 2009; Bradic *et al.*, 2012). A general drop of diversity in the subterranean ecomorph is characteristic of both sites, but the factors causing this drop may not be the same, as differences between regions are ample. For example, the strength of selection following invasion of the subterranean environment was probably higher in Romania, where the groundwater is sulphidic. Also, both ecomorphs are genetically much more diverse in Romania than in Slovenia. This is probably a consequence of the geographical setting of the Romanian site close to the Danube, as large rivers were shown to harbour the highest genetic diversity for this species (Verovnik *et al.*, 2005). The interesting question is now whether a 50% difference in ancestral genetic variation affects the evolutionary potential of the respective descendant cave population. If so, one could expect less morphological traits undergoing significant change in the Slovenian ecomorph pair. The opposite is true – 68% of all analysed traits changed in Slovenia and 46% in Romania, suggesting that the degree of phenotypic evolution does not reflect ancestral genetic variability.

Morphological evolution in the subterranean habitat in a comparative framework

Convergent traits of subterranean animals are likely to have evolved under the specific selective regime of the subterranean habitat (Christiansen, 1961; Jones *et al.*, 1992; Trontelj *et al.*, 2012). Following this premise, we can pick 18 potential candidate traits for further examination of their function in both *A. aquaticus* ecomorph pairs; 10 are elaborated changes and the rest are reduced. The adaptive function of most of them is unknown. Exceptions are antennal length increase and eye reduction, possibly increasing sensory and neurological or energetic efficiency, respectively (Culver *et al.*, 1995). The extent to which natural selection is operating in the process of pigment and eye reduction is still unclear. Existing explanations for eye and pigment loss in cave animals include drift, selection and pleiotropy (Culver, 1982; Wilkens, 1988; Jeffery, 2005). In *Asellus*, accumulation of mutations by genetic drift, as well as alternate scenarios involving direct or indirect (pleiotropy) selection were proposed in the pathways responsible for reduction of eyes and body pigmentation (Protas *et al.*, 2011). The biochemical mechanism of pigment loss and the type of pigment itself are unclear, but it seems that it is ommochrome rather than melanin (Protas & Jeffery, 2012). The adaptive value, if any, of shortening and simultaneous multiplication of some setae on only specific body parts in the subterranean *Asellus*, remains enigmatic.

Several traits changed only in one pair of ecomorphs, and two traits changed in opposite direction in both pairs. Factors responsible for this pattern of character change might include random drift in one or both populations, or specific characteristics of the selective environment of the Slovenian or Romanian subterranean habitat (Kaeuffer *et al.*, 2012). Drift, as opposed to selection, has been proposed for subterranean populations because of their small size (Crouau-Roy, 1989). An obvious candidate for traits undergoing specific selection regimes is the proportion of the fifth pleopod that determines the size of the respiratory area, which, in turn, is important in the process of osmoregulation (Prevorčnik *et al.*, 2009). As the Romanian subterranean water is rich in sulphide and chlorine, nonconvergence of this trait could be a consequence of adaptation to special ecological conditions (Appendix S1). The observed opposite change in marginal setae number on the pleotelson, however, might be a size-related trait resulting from the increased overall size of the Romanian subterranean ecomorph. Namely, setae density calculated as number of marginal setae vs. pleotelson length did not change significantly in the Romanian ecomorph pair.

An often mentioned hypothesis is that morphological variability of some traits should increase as a consequence of released selection in the subterranean environment (Kosswig & Kosswig, 1940). We could find no convincing support for this hypothesis in the compared pairs of ecomorphs. Both traits with increased variance include the number of setae on certain pereopods, whose function is unknown. Even if the change can be attributed to relaxed selection, this hypothesis cannot be generalized to other subterranean populations, as the traits changed only in the subterranean ecomorph of the Romanian pair.

An alternative view of convergence and parallelisms in subterranean evolution

Our example shows that only a few of all evolving traits consistently undergo convergent or parallel evolution, albeit the most conspicuous ones – eye loss, depigmentation, elongation of some appendages. This happens regardless of (i) nutrient availability, (ii) habitat type (sinking river open to the surface vs. closed aquifer), (iii) water chemistry (sulphidic vs. nonsulphidic freshwater), (iv) ancestral genetic variability and (v) population genetic structure of cave populations (structured vs. panmictic). With respect to those traits, our results confirm the century-long paradigm of convergent evolution in caves. What is more, they do not contradict the new 'darkness-as-major-selective-force' hypothesis by Culver *et al.* (2010), as narrow-sense troglomorphies seem unaffected by differences in nutrient availability.

However, less studied traits, as well as overall morphological similarity (eye loss and depigmentation excluded), follow divergent pathways in the Romanian and Slovenian subterranean lineage, supporting Pipan & Culver's (2012) recent critique. As new types of subterranean habitats – such as sulphidic caves, shallow subterranean habitats (Culver & Pipan, 2014) and even specific micro-niches within a single habitat (Fišer *et al.*, 2012) – are becoming better known, traits usually considered as convergent turn out to undergo nonconvergent evolution. The new model of morphological evolution in caves is therefore likely to become one of a mosaic of convergent and divergent traits reflecting differences between and heterogeneity within subterranean environments.

Acknowledgments

This research was supported by the Slovenian Research Agency as part of the first author's PhD project through contract no. 1000-09-310036, and through Research Program P1-0184. We would like to thank Mihai Baciu, Gregor Bračko, Traian Brad, Teo Delić, Cene Fišer, Žiga Fišer, Špela Gorički, Jure Jugovic, Ajda Moškrič, Dumitru Pegulescu, Boris Sket, Maja Zagmajster and Valerija Zakšek for providing samples and helping with field work. We are grateful to Maja Jelenčič and Tomaž Skrbinšek for help with microsatellites, and Cene Fišer, Maja Zagmajster and Valerija Zakšek for comments on an earlier version of the manuscript. David Culver and two anonymous reviewers provided further valuable comments and constructive criticisms.

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Supporting information

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

Appendix S1 Samples and habitat characteristics.

Appendix S2 Sampling localities, DNA sequence data, GenBank accession numbers, and microsatellite data.

Appendix S3 Morphological traits and their evolutionary change.

Appendix S4 Nuclear phylogeny and mtDNA haplotype networks.

Data deposited at Dryad: doi: 10.5061/dryad.23j81

Received 12 September 2014; revised 19 February 2015; accepted 20 February 2015